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(54) Title: USE OF RADIOLABELED MONOCLONAL IgM IN THERAPY FOR CANCER AND AUTOIMMUNE DISEASE

(57) Abstract

It has been discovered that large antibody aggregates or molecules, such as IgM or conjugated IgG or IgG fusion proteins, can be used to treat tumors by intracompartmental or intratumoral administration of anti–tumor antibody coupled to a toxin. The method can also be used in the treatment of certain disorders characterized by deposition of immune complex, for example, rheumatoid arthritis. In the preferred embodiment, the antibody is IgM and the toxin is a radioisotope, most preferably ^{111}In–labelled IgM or ^{90}Y–labelled IgM. Examples demonstrate effectiveness in mice models.

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USE OF RADIOLABELED MONOCLONAL IGM IN THERAPY FOR CANCER AND AUTOIMMUNE DISEASE

The present invention is generally in the area of cancer and autoimmune disease therapy using radiolabeled IgM antibodies specific for tumor or antibody producing cells.

The United States government has certain rights in this invention by virtue of National Institutes of Health grants CA 51161 and CA 16672.

Background of the Invention

Cancer is a major health problem. Two populations of human cancer patients are highlighted as examples.

Head and Neck Cancer

Each year in the United States there are approximately 53,000 new cases of cancer of the head and neck. Parker, et al., CA Cancer J. Clin. 65: 5-27 (1996). Over 90% are squamous cell carcinomas that originate on the mucosal surface of the upper aerodigestive tract. Shah, et al. CA Cancer J. Clin. 45: 352-368 (1995). At the time of presentation fewer than 10% of patients have distant metatases and only one-third of patients have small primary tumors less than four cm diameter that can be cured with local radiation therapy or surgery. Haraf, et al., Head and Neck Cancer: Basic and Clinical Aspects, pp. 173-198, Editors W.K. Hong and R.S. Weber, Kluwer, Norwell, MA (1994). Advanced disease, which is characterized by large primary tumors and regional lymph node involvement, often requires higher doses of radiation for cure than can be tolerated by the surrounding normal tissues and while bulky disease can be removed by radical surgery, the procedure is often mutilating and accompanied by problems associated with prolonged rehabilitation and residual morbidity.

Radiation therapy and surgery are frequently combined for patients with advanced disease, but 60% of them will still experience a local recurrence. Tupchong, et al. Int. J. Radiat. Oncol. Biol. Phys. 20: 21-28, (1991).

Ovarian Carcinoma

Ovarian carcinoma is the fourth most common cause of death from cancer in women, with 14,200 deaths annually in the U.S. (146Neijt, N. Engl. J. Med. 334, 50-51 (1996); Parker, et al., CA Cancer J. Clin. 199, 47:5-27 (1997)). The 5-year survival rate ranges from 50% for patients with tumors confined to the ovaries to less than 20% for patients with stage 3 and 4 disease (Teneriello, CA Cancer J. Clin. 45:71-87 (1995). At diagnosis, 75-85% of all patients have advanced disease (McGuire, Cancer (suppl) 71:1541-150 (1993)). Current first-line therapy for these patients consists of cytoreductive surgery followed by i.v. platinum-based chemotherapy that now includes paclitaxel. Response rates range from 60%-80% (McGuire, et al., N. Engl. J. Med. 334:1-6 (1996); Christian and Trible Gynecol. Oncol. 55:S143-S150 (1994); Thigpen, et al., Semin. Oncol. 18:248-154 (1991)); however, most of these patients will relapse.

The observation that most patients have disease in the peritoneal cavity and that it is the most common site of relapse has focused attention on regional treatment approaches. Intraperitoneal chemotherapy, i.p. colloidal ^{32}P, and external beam radiotherapy of the entire abdomen have been reported (Markman, Semin. Oncol. 18, 248-254 (1991); Ozols, et al., Epithelial ovarian cancer. In: Principles and Practice of Gynecologic Oncology. Hoskins, et al., (eds.) (J.B. Lippincott, Philadelphia, 1992) pp. 731-781). Tumor response and prolongation of survival are limited to patients with microscopic or very small macroscopic disease. Tissue penetration by i.p. administered chemotherapeutic agents is confined to the outer 1-2 mm of the tumor surface (Los, et al., Cancer Res. 49, 3380-3384 (1989); Ozols, et al., Cancer Res. 39, 3209-3214 (1979)). Most chemotherapy drugs have short half-lives in the peritoneal cavity, which is directly proportional to their small molecular size (Dedrick, et al., Cancer Treat. Rep. 62:1-11 (197)). Intraperitoneal colloidal ^{32}P nonselectively irradiates the surface of the peritoneal cavity (Boye, et al., Br. J. Radiol. 57:395-402 (1984); Currie, et al., Gynecol. Oncol. 12:193-218 (1981)). It is also prone to aggregation that produces inhomogenous dose distributions (Kaplan, et al., Radiology 138:683-688 (1981)). The presence of

radiosensitive organs in the abdominal region prevents the delivery of high doses of external beam radiation. The probability of eradicating measurable disease (cancer nodules greater than or equal to 5 mm) with low-dose external beam radiation is small.

Chemoresistant malignant cells usually respond to radiation and prior radiation therapy does not induce radioresistance. Intraperitoneal administration of murine IgG radiolabeled with ^{90}Y has demonstrated efficacy as an adjuvant (Stewart, et al., J. Clin. Oncol. 1941-1950 (1990); Maraveyas, et al., Cancer (suppl) 73:1067-1075 (1994)). However, myelotoxicity prevented dose escalation beyond 30 mCi of activity.

New applications of radioimmunotherapy can make an important contribution to the solution of the oncological problems highlighted in these examples.

Radioimmunotherapy (RIT)

RIT employs an antibody that recognizes a tumor-associated antigen to selectively deliver therapeutic radiation to a tumor. The specificity and high affinity that antibodies display towards their antigens led to optimistic expectations that radiolabeled antibodies would be "magic bullets" for cancer with very high therapeutic ratios. Pressman D., The Handbook of Cancer Immunology, Waters H (ed), Vol 5, 30-50 (Garland STPM Press, New York, 1978). This initial enthusiasm ignored the pharmacologic restraints imposed upon delivering such large molecules through the blood stream to tumors. Consequently, clinical RIT trials, particularly those for solid tumors, have been disappointing. Research has continued in order to understand the limitations of these reagents and to develop improved reagents and methodologies. RIT is now on the verge of making a large impact in the systemic treatment of hematologic malignancies, but it remains ineffective in the treatment of solid tumors.

Systemic or Intravenous RIT

Intravenous (i.v.) administration of radiolabeled murine IgG for treatment of solid tumors has been limited by low tumor deposition of radioactivity, myelotoxicity and induction of human anti-mouse antibodies (HAMA). Deposition of radioactivity in tumors following i.v. administration is frequently in the range of between 0.001 and 0.01% injected dose per gram (%ID/g), which rarely produces a tumor response. Epenetos, et al., Cancer Res. 46: 3183-3191(1988). Myelotoxicity is frequently the dose-limiting normal tissue toxicity after i.v. RIT through irradiation of bone marrow cells by blood borne immunoconjugate. Stein, et al., Br. J. Haematol. 80: 69-76, (1992); Vriesendorp, et al., Exp. Hematol. 24, 1183-1190 (1996). Most monoclonal antibodies used clinically are of murine origin and frequently induce the formation of human anti-mouse antibodies (HAMA) and an anti-antibody response in immunocompetent patients. Radioimmunoconjugates upon readministration can be bound by the anti-antibodies and prevented from reaching tumor. Additionally, these immune complexes are rapidly cleared by the reticuloendothelial system which may significantly increase the radiation dose to the liver and spleen. Immunosuppression and plasmapheresis of patients have been introduced to circumvent the induction of HAMA, but with limited success.

Systemic RIT utilizing radiolabeled IgG for hematologic malignancies has been more successful than RIT for solid tumors. However, the same limitations apply: low tumor targeting, myelotoxicity and to a lesser extent HAMA formation. Promising results have been obtained with systemic RIT for patients with Hodgkin's disease (Tupchong, et al. Int. J. Radiat. Oncol. Biol. Phys. 20, 21-28-1991; Vriesendorp, et al., Cancer Res. 55(suppl):5888s-5892s (1995)) with B-cell lymphomas (Vriesendorp, et al., Exp. Hematol. 24, 1183-1190 (1996), Wahl, et al., J. Nucl. Med. 28, 1736-1744 (1987), Waldmann, et al., Metabolism of immunoglobulins In: Progress in Allergy, 13, p. 35 Editors: Kallos, et al., (Karger, NY 1969), and Weinstein, et al., Science 222, 423-426 (1983)) and with T-cell lymphomas (Wheldon, Nucl. Med. Commun. 14, 408-410 (1993)).

Intracompartmental RIT

In view of the low level of tumor response following systemic RIT for patients with solid tumors, intracompartmental RIT has been attempted using radiolabeled IgG or its fragments. Small numbers of human cancer patients with a variety of solid tumors, such as in colon cancer, glioma, and ovarian cancer have been treated through the direct administration to the tumor or physical cavity that contains tumor. The total response rate for the combined quoted studies was 31%. Riva P. et al., Int J Biol Markers 8: 192-197 (1993). Papanastassiou V. et al., Br J Cancer 67: 144-151 (1993). Finkler N.J. et al., Gynecol Oncol 34: 339-344 (1989).

Even though intracompartmental administration of radiolabeled murine IgG can produce higher tumor deposition of radioactivity (Schlom, et al., Cancer Res. (Suppl) 50, 820s-827s (1990), Malamitsi, et al., J. Nuc. Med. 29, 1920-1925 (1988), and Haisma, et al., Am. J. Obstet. Gynecol. 159, 843-848 (1988)) and higher rates of tumor responses than i.v. administration, deposition of radioactivity in tumors remains low, myelotoxicity occurs (Hopkins, et al., Radiother. Oncol. 34, 121-131 (1995), Maraveyas, et al., Cancer (suppl) 73, 1067-1075 (1994), Hird, et al., Br. J. Cancer 68, 403-406 (1993), Breitz, et al., J. Nucl. Med. 36, 754-761 (1995)) and HAMA are induced (Riva, et al., Init. J. Biol. Markers 8, 192-197 (1993), Finkler, et al., Gynecol. Oncol. 34, 339-344 (1989), Jacobs, et al., Obstet. Gynecol. 82, 586-593 (1993), Stewart, et al., J. Clin. Oncol. 8, 1941-1950 (1990), Maraveyas, et al., Cancer (suppl) 73, 1067-1075 (1994), and Hird, et al., Br. J. Cancer 68, 403-406 (1993)).

It is therefore an object of the present invention to provide a means for more effective treatment of solid tumors.

It is a further object of the present invention to provide a means to lower immunogenicity compared to non-human antibodies and decrease myelotoxicity compared to treatment with anti-tumor IgG.

It is another object of the present invention to provide a means for treating solid tumors locally or intracompartmentally with high levels of deposition of radiation in the tumors.

Summary of the Invention

It has been discovered that large antibody aggregates or molecules, such as IgM or linked IgG (dimers, trimers, etc.) or IgG fusion proteins, can be used to treat tumors by intracompartmental or intratumoral administration of anti-tumor antibody coupled to a toxin. In the preferred embodiment, the antibody is IgM and the toxin is a radioisotope, most preferably ^{111}In-labelled IgM for diagnostic or dosimetric purposes or ^{90}Y-labelled IgM for therapeutic purposes.

Examples demonstrate effectiveness in mice models, and include protocols for treatment of peritoneal carcinomatosis and recurrent or persistent Hodgins' Disease, Kaposi Sarcoma, and head and neck cancer.

The antibody conjugates can also be used to treat autoimmune disease characterized by production of antibodies that produce immune complexes at defined surfaces, for example, Rheumatoid arthritis, where the conjugates diffuse into the joints to provide relief from the inflammation associated with the disorder.

Brief Description of the Drawings

Figure 1 is a graph of tumor response following i.l. administration of ^{90}Y-labeled CR4E8, as a function of tumor volume (mm^3) and the amount of radioactivity which is administered.

Figure 2 is a graph of the occurence of moist desquamation in mice treated with ^{90}Y-labeled CR4E8, as a function of initial tumor volume (mm^3) and the amount of radioactivity which is administered.

Figure 3 is a graph of the incidence of tumor recurrence in mice treated with i.l. ^{90}Y-labeled CR4E8, as a function of initial tumor volume (mm^3) and the amount of radioactivity which is administered.

Detailed Description of the Invention

The failure of i.v. RIT to effectively treat solid tumors stems primarily from the route of administration and the size and species of the immunoglobulin. Intravenous administration places a large amount of radioimmunoconjugate into the blood where it is diluted and distributed

throughout the circulatory system. Immunoglobulins are large molecules (150-950 kDa) that move primarily by convection (Jain, Scientific American 271, 58-65 (1994)). Tumors frequently have high interstitial pressure, which prevents the radioimmunoconjugates from entering the tumor interstitium by convection.

The limitations of systemic administration of IgG are avoided by (1) using intralesional or intracompartmental administration of antibody and (2) selection of an antibody that delivers a high dose of toxin, preferably radioisotope, and, due to the antibody's large size and affinity for tumor cells, stays in the general vicinity of where it is administered, as well as is targeted to the tumor.

IgG not conjugated to antigen, in circulation or accidentally deposited in perivascular spaces in patients with poor venous access, has a short half-life due to rapid catabolism and disappearance of small radioactivity moieties via circulation and urine elimination. IgG bound to antigen in blood or in tumor or IgM bound to tumor antigens is catabolized much slower. This underlines the potential of antigen specific IgM as a carrier for radiation. Unmodified IgM in circulation has a half-life of only five days, less than two half-lives of yttrium-90. The decrease in IgM catabolism by antigen binding allows for the delivery of more radiation and higher clinical efficacy. Simultaneously the tumor binding of IgM also keeps the radiation limited to the location where it is needed.

The best way to achieve high tumor deposition of radioactivity and avoid systemic toxicity, is to directly administer the radioimmunoconjugate at the disease site. This route of administration is typically applied to diseases that stay confined over their clinical history to a compartment. In this application, a compartment is defined as either a physical space like the peritoneal or pleural cavities or a large primary tumor. The intralesional (i.l.) or intraperitoneal (i.p.) administration of radiolabeled IgM provides much higher levels of radioactivity in tumors. Myelotoxicity is avoided since the intralesionally or intracompartmentally administered radioimmunoconjugate remains localized in the tumor or compartment. Initial clinical trials utilizing radiolabeled human IgM for diagnostic

purposes have demonstrated its low degree of immunogenicity. De Jager, R., et al., Sem. Nucl. Med. 23: 165-179, (1993). Other applications include diseases where a large tumor could be treated palliatively.

The reactivity of the antibody with tumor cell antigens coupled with the high avidity (10 antigen binding sites) and large mass of IgM (900 kDa) keeps the radioimmunoconjugate within the tumor. Because IgM is soluble, the radioimmunoconjugate should diffuse through the tumor over time. Thus, more selective high-dose radiation of irregular tumor volumes is possible with i.1. RIT than with conventional radiation therapy.

RIT can result in a mixture of immunological and radiation effects. Studies in patients with Hodgkin's disease clearly established the radiation aspects of RIT. Tumor masses that are not targeted do not shrink. Dose escalation increases the chance for a tumor response. Small fraction sizes are not effective. Tumor recurrences after RIT are not radioresistant. All these aspects are similar to observations made over the years in patients undergoing external beam radiation. This indicates that RIT has an effect based on and eqiuvalent to external beam radiation but in another form. Any pathological process in the body which is susceptible to radiation but cannot be treated successfully with it due to toxicity concerns, can therefore be treated with RIT, which by definition has a higher therapeutic ratio than other non specific delivery systems of radiation. Examples of other disorders include rheumatoid arthritis and restenois, as discussed in more detail below.

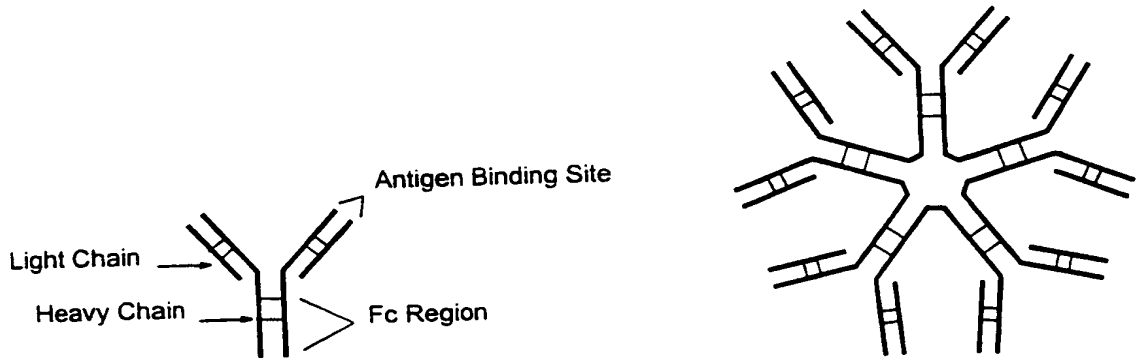
Components of RIT

In vitro

Several components determine the design and synthesis of a therapeutic radioimmunoconjugate: tumor antigen, an antibody that is immunoreactive with the tumor antigen, a cytotoxic agent such as a radioisotope and means for coupling the cytotoxic agent to the antibody which does not alter immunoreactivity of the antibody. These *in vitro* components are discussed below, followed by a discussion of the variables that influence the *in vivo* distribution of the radioimmunoconjugate after administration.

Antibodies

There are five classes of antibodies: IgA, IgD, IgE, IgG, and IgM. Of these, only IgG and IgM have been used as radioimmunoconjugates. This is, in part, due to technical reasons since splenic B-cells from mice generate hybridomas that usually secrete IgG_1 and IgG_{2a} (Keenan, et al., J. Nucl. Med. 26:531-537 (1985)) while hybridomas produced from human lymphocytes frequently secrete IgM. IgG and IgM are very similar in structure and function. IgG is a monomer, while IgM is a pentamer composed of five identical monomers. Each monomer is composed of two identical heavy chains and two identical light chains. The light chains are composed of two domains: the variable domain on the amino terminus, which contains the antigen-binding site, and a constant domain on the carboxy terminus. The heavy chain has a similar arrangement with one variable domain on the amino terminus end followed by three constant domains for IgG and four constant domains for IgM. Each heavy chain is bound to a light chain through disulfide bonds. This association produces a more effective antigen-binding site that gives the antibody its specificity. The last two constant domains of the heavy chain contain the Fc region, which regulates effector functions of the antibody. Two strands composed of a light chain and a heavy chain are linked together through the heavy chains by disulfide bonds to form the monomer. IgM possesses an additional polypeptide chain, the J chain, that is thought to assist the polymerization of the pentamer.



IgG

~150 kDa

IgM

~950 kDa

Although described primarily with reference to IgM, it is to be understood that other antibody molecules can be substituted for IgM,

including conjugates or polymers of IgG, immunoglobulin fusion proteins, and recombinant fragments or humanized IgM antibodies, can be delivered to a site or compartment where the antibody molecules will remain localized. These are collectively referred to herein as "IgM" unless specifically noted.

Polyclonal Antibodies

IgG and IgM can be isolated from the sera of immunized animals. These antibodies are termed polyclonal in that they arise from many different clones of antigen-stimulated B-cells. The percentage of the polyclonal antibodies that is reactive with the antigen used for immunization is low, (less than 20%). Affinity purification is sometimes used to increase this percentage. Polyclonal antibodies are now used infrequently since it takes several months of immunizations to generate antibodies of sufficient specificity and titer, and there is lot-to-lot variability.

Monoclonal Antibodies

The introduction of hybridoma technology allowed the large-scale production, through cell culture techniques, of monoclonal antibodies with defined specificity and very high reactivity. Most monoclonal antibodies developed for clinical use are murine in origin owing to the initial requirement of splenic tissue as a source of stimulated B-cells. Mouse monoclonal antibodies frequently induce HAMA in immunocompetent patients. Many attempts have been made at reducing the adverse effects of HAMA. Some are pharmacological like plasmapheresis (Zimmer, et al., J. Nucl. Med. 29:174-180 (1988)) and immunosuppresion of the patient (Riva, et al., Int. J. Biol. Markers 8:192-197 (1993); Weiden, et al., Cancer 73:1093-1097 (1994)). Others use enzymes like pepsin and papain to cleave the Fc region from IgG to produce smaller fragments for administration that retain the ability to bind antigen but lack the effector portion of the immunoglobulin (Carrasquillo, et al., Cancer Treat. Rep. 68:317-328 (1984)). More recently, genetic engineering has been employed to replace immunogenic regions of a murine antibody with human regions. In the first attempts, the constant domains of a mouse antibody were replaced with the constant domains of a human antibody (Boulianne, et al., Nature 312:643-646 (1984); Morrison, et al. Proc. Natl. Acad. Sci. 81:6851-6855 (1984)).

The resulting antibodies are termed chimeric antibodies and can still be immunogenic (Khazaeli, et al., Cancer Res. 51:5461-5466 (1991)). Further refinements through the grafting of the hypervariable regions of a mouse antibody onto the variable domains of a human antibody have produced what are termed humanized antibodies that may possess reduced immunogenicity (Jones, et al., Nature 321:522-525 (1986)).

Human antibodies are the preferred choice as the least immunogenic antibody for human patients (De Jager, et al. Semin. Nucl. Med. 23:165-179 (1993)). Additionally, human antibodies may be less reactive with normal human tissue antigens and be more specific for human tumor antigens. The realization that patients with malignancies may have stimulated B-cells in their tumor-draining lymph nodes and in their peripheral blood that produce immunoglobulins against their cancer has provided an important source of human B-cells (Freedman, et al., Hybridoman 10:21-33 (1991); Chen, et al., Hum. Antibodies Hybridomas 5:131-142 (1994); Nishinaka, et al., Cancer Res.56:5666-5671 (1996)). After fusion with a myeloma cell, many human hybridomas are unstable and produce only low titers of immunoglobulins. Production problems may be overcome by genetic engineering techniques such as the construction of yeast artificial chromosomes and their expression in cell culture or transgenic animals (Pennisi, Sci. News 143:360-363 (1993)).

Antigens

The tumor antigens used to target radioimmunoconjugates are almost exclusively tumor-associated antigens. These are not specific markers for tumor cells in most cases; rather, they are overexpressed on tumor cells compared with normal tissue, or they are found in association with normal fetal tissue, such as CEA (Gold, et al., J. Exp. Med. 122, 467-481 (1965)), AFP (Abelev, Adv. Cancer Res. 14, 295-350 (1971)) or with normal progenitor cells of that organ in the adult (CEA). Tumor antigens can be localized in the tumor interstitium, on the tumor cell membrane, or in the tumor cell cytoplasm or nucleus.

The location of the tumor antigen directs the selection of the route of administration of the radioimmunoconjugate and the type of therapeutic

radioisotope needed for effective tumor sterilization. Antigens that are found on leukemia cells in circulation and antigens expressed on tumor neovasculature are readily accessible to i.v. administered reagents. Antigens that are expressed on the surface of tumor cells are readily accessible to i.l. or i.p. administered radioimmunoconjugates. Antigens secreted into the tumor interstitium are most accessible to i.l. administration.

The preferred anti-tumor antibodies are human IgM that recognize tumor associated antigens that are either expressed on the tumor cell membrane or secreted into the tumor interstitium. Table 1 lists the preferred antibodies, disease applications, and sources.

TABLE 1: ANTIBODIES FOR USE IN IgM RIT.

Designation	Origin	Antigen size/location	Disease application
AC6C3-2B12[1]	human	32 kDa cell membrane (SKOV3, human ovarian cell line)	Adenoma (breast, colon, ovarian)
CR4E8[2]	human	55 kDa cell membrane (SW756, human cervical carcinoma cell line)	Squamous cell (cervical, head carcinoma and neck) prostate
ACC 0101	murine ferritin (440 kDa), interstitium		Hodgkin's disease, head and neck, hepatoma, sarcoma
Kaposi's			
L72[3]	human GD_2 (ganglioside) cell membrane		glioma, melanoma, neuroblastoma
126-4[4]	murine GD_2 (ganglioside) cell membrane		glioma, melanoma, neuroblastoma

References:

1 Freedman RS, Ioannides CG, Tomasovic B, et al. Development of a cell surface reacting human monoclonal antibody recognizing ovarian and certain other malignancies. Hybridoma, 1991, 10: 21-33.

2 Chen P-F, Freedman RS, Chernajovsky Y, et al. Amplification of immunoglobulin transcripts by the non-palindromic adapter polymerase chain reaction (NPA_PCR). Nucleotide sequence analysis of two human monoclonal antibodies recognizing two cell surface antigens expressed in ovarian, cervix, breast, colon and other carcinomas. Hum Antibod Hybridomas, 1994; 5: 131-142.

3 Katano M, Jien M, Irie Reiko. Human monoclonal antibody to ganglioside GD2-inhibited human melanoma xenograft. 1984; Eur J Cancer Clin Oncol, 20; 1053-1059.

4 Schulz G, Cheresh DA, Varki NM, et al. Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res, 1984, 44: 5914-5920.

Cytotoxic Compounds: Radioisotopes

Although described primarily with reference to radioisotopes, especially indium ("In"), which is useful for diagnostic purposes, and yttrium ("Y"), which is cytotoxic, other substances which kill cells can be substituted for the radioisotope. The radioisotopes are preferred since they are small and well characterized, and can be used as diagnostics and followed after administration using standard non-invasive radioimaging techniques. Non-radioisotopes can include toxins and substances which elicit the host to attack the tumor cells, as well as synthetic or natural chemotherapeutic drugs (Halpern, et al., J. Nucl. Med. 29:1688-1696 (1988); Quadri, et al., Nucl. Med. Biol. 20:559-570 (1993); Wang, et al., Radiat. Res. 141:292-302 (1995)), oligonucleotides (Mujoo, et al., Oncogene 12:1617-1623 (1996)), cytokines (Markman, Semin. Oncol. 18:248-254 (1991); Dedrick, et al., Cancer. Treat Rep. 62:1-11 (1978)), and radioactive colloids (Rowlinson, et al., Cancer Res. 47:6528-6531 (1987)). These can be conjugated to the antibody using standard chemical techniques, or in some cases, using recombinant technology, for example, fusion proteins.

As radioisotopes decay, they emit characteristic photons or particles or both. Photons, commonly referred to as gamma rays, are penetrating. If their energy level is high enough, they can travel through the body and be detected by diagnostic instrumentation. Radioisotopes that emit photons can be attached to an antibody and used for diagnostic imaging. This application is termed radioimmunoscintigraphy (RIS).

The shorter the distance between the antigen and the target (tumor cell DNA), the shorter the required range of emission of the radioisotope. Auger electrons have a very short path length (5-10 nm) and need to be internalized to be cytotoxic (Adelstein, et al., Nucl. Med. Biol. 14:165-169 (1987)). Only antibodies that are internalized after binding to a cell should be considered for radioisotopes that emit Auger electrons. Alpha particles need to be close to a cell (within 3-4 cell diameters) to be effective (Vriesendorp, et al., Radioimmunoglobulin therapy. In: High Dose Cancer Therapy. Armitage, et al. (eds). (Williams & Wilkins, Baltimore, MD 1992) pp. 84-123), so antibodies that target interstitial antigens would not be

desirable. Both Auger electrons and alpha emitters have high selectivity because their short-range emission will not irradiate neighboring normal cells. Conversely, untargeted neighboring malignant cells will also avoid irradiation. Tumor sterilization by radioimmunoconjugates carrying Auger electron or alpha emitters can only be achieved by the targeting of all clonogenic tumor cells.

Long-range beta emissions are advantageous as they will produce more homogenous distribution of radiation within a tumor, preventing "cold" spots by lethally irradiating untargeted cells through cross-fire of emissions from neighboring areas (Wheldon, Nucl. med. Commun. 14:408-410 (1993)). The drawback of long-range emissions is that they are less selective and less effective for treating tumor masses that are smaller than the path length of the beta particles. With smaller masses, most of the energy is deposited outside of the tumor mass. This increases normal tissue toxicity and reduces the tumor dose.

The radiometals ^{111}In and ^{90}Y are, respectively, pure γ- and pure β-emitters. Iodine-125, the most commonly used emitter of Auger electrons, has a half-life of 60 days and frequently is released by the immunoconjugate *in vivo* (dehalogenation) (Vriesendorp, et al., 1992). The most commonly considered alpha emitters for clinical use, astatine-211 and bismuth-212, have short half-lives (7.2 h and 1.0 h, respectively) and decay into radioactive isotopes, that may not be retained by the immunoconjugate after the first alpha emission (Wilbur, Antibiot. Immunoconjug. Radiopharm. 4:85-97 (1991)). The use of an immunoconjugate radiolabeled with ^{111}In has been proposed to predict the behavior of the poorly imageable ^{90}Y-labeled immunoconjugate (Korngold, et al., Cancer Res. 20:1488-1494 (1960); Welt, et al., J. Clin. Oncol. 12:1561-1571 (1994); Breitz, et al., J. Nucl. Med. 33:1099-1112 (1992); Vriesendorp, et al., Cancer Res. (suppl) 55:5888s-5892s (1995)). Previous studies using stable radiometal chelation have demonstrated similar biodistributions for radioimmunoconjugates labeled with ^{111}In and ^{90}Y (Welt, et al., J. Clin. Oncol. 12:1561-1571 (1994); Breitz, et al., J. Nucl. Med. 33:1099-1112 (1992)). In practice, immunoscintigraphy of patients after administration of the ^{111}In-labeled antibody would allow the

physician to screen patients for tumor localization and determine the *in vivo* stability of the radioimmunoconjugate. Then, only patients expected to benefit from treatment would receive the antibody again, which is now labeled with a therapeutic quantity of ^{90}Y. This treatment approach would allow patients to be treated as outpatients because the administered activity of ^{111}In is low and the emissions from ^{90}Y are largely absorbed in the vicinity of the tumor.

Beta-emitting isotopes for RIT can be either mixed beta and gamma emitters like ^{131}I, with 90% gamma, and ^{67}Cu, with 60% gamma (Schubiger, et al., Bioconjug. Chem. 7:165-179 (1996)) or pure beta-emitters like ^{90}Y and ^{32}P. Both types of radioisotopes have their supporters. Advocates of radioisotopes with mixed emissions like the simplicity of using the same radioimmunoconjugate at a low activity to demonstrate tumor targeting with gamma camera imaging and, by escalating the activity, administer therapy in the next treatment. This way, the pharmacokinetics of the radio-immunoconjugate should be identical for both administrations, and the first administration should be predictive for the second administration if the protein dose is held constant. However, this simplicity comes at the cost of exposing patients to high doses of gamma rays when therapeutic activities are employed. In addition, elaborate inpatient management becomes necessary to prevent the exposure of medical personal and the general public to unacceptable levels of radiation.

Attachment of Cytotoxic compound or Radioisotope to Antibody

Some radioisotopes can be attached directly to the antibody; others require an indirect form of attachment. The radioisotopes ^{125}I, ^{131}I, ^{99m}Tc, ^{186}Re and ^{188}Re can be covalently bound to proteins (including antibodies) through amino acid functional groups. For radioactive iodine it is usually through the phenolic group found on tyrosine. There are numerous methods to accomplish this: chloramine-T (Greenwood, et al. Biochem J. 89: 114-123 (1963)); and Iodogen (Salacinski, et al. Anal. Biochem. 117: 136-146 (1981)). Tc and Re can be covalently bound through the sulfhydryl group of cysteine (Griffiths, et al. Cancer Res. 51: 4594-4602 (1991)). The problem with most of the techniques is that the body has efficient methods to break

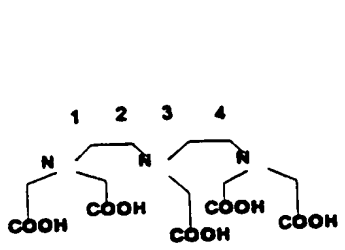
these covalent bonds, releasing the radioisotopes back into the circulatory system. Generally, these methods are acceptable for imaging purposes (^{99m}Tc), but not for therapeutic purposes.

Numerous types of cytotoxic compounds can be joined to proteins through the use of a reactive group on the cytotoxic compound or through the use of a cross-linking agent. A common reactive group that will form a stable covalent bond in vivo with an amine is isothiocyanate (Means, et al. Chemical modifications of proteins (Holden-Day, San Francisco 1971) pp. 105-110). This group preferentially reacts with the ε-amine group of lysine. Maleimide is a commonly used reactive group to form a stable *in vivo* covalent bond with the sulfhydryl group on cysteine (Ji. Methods Enzymol 91: 580-609 (1983)). Monoclonal antibodies are incapable of forming covalent bonds with radiometal ions, but they can be attached to the antibody indirectly through the use of chelating agents that are covalently linked to the antibodies. Chelating agents can be attached through amines (Meares, et al., Anal. Biochem. 142:68-78 (1984)) and sulfhydral groups (Koyama Chem. Abstr. 120:217262t (1994)) of amino acid residues and also through carbohydrate groups (Rodwell, et al., Proc. Natl. Acad. Sci. 83:2632-2636 (1986); Quadri, et al., Nucl. Med. Biol. 20:559-570 (1993)). Since these chelating agents contain two types of functional groups, one to bind metal ions and the other to joining the chelate to the antibody, they are commonly referred as bifunctional chelating agents (Sundberg, et al., Nature 250:587-588 (1974)).

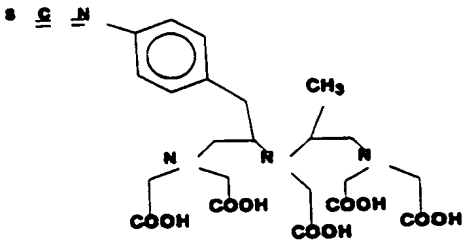
Crosslinking agents have two reactive functional groups and are classified as being homo or heterobifunctional. Examples of homobifunctional crosslinking agents include bismaleimidohexane (BMH) which is reactive with sulfhydryl groups (Chen, et al. J Biol Chem 266: 18237-18243 (1991) and ethylene glycolbis[succinimidylsucciate] EGS which is reactive with amino groups (Browning, et al., J. Immunol. 143: 1859-1867 (1989)). An example of a heterobifunctional crosslinker is m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Myers, et al. J. Immunol. Meth.121: 129-142 (1989)). These methodologies are simple and are commonly employed.

^{111}In and ^{90}Y are available as trivalent ions in aqueous hydrochloric acid (pH 1). They precipitate as insoluble hydroxides at pH greater than 3.4 for In and greater than 7 for Y (Brunner, et al., Radiometals and their chelates. In: Principles of Nuclear Medicine. Wagner, et al., (eds.) (2nd ed., Saunders, Philadelphia 1995)). Trivalent indium can be stabilized at neutral pH if a chelate with at least 6 donor ligands is used to saturate the coordination sites of the ion. The ionic radius of trivalent yttrium is 15% larger than that of indium and requires a chelating group that can donate at least 8 ligands. The polyamino-polycarboxylic ligand, diethylenetriaminepentaacetic acid (DTPA) has 8 ligands and will strongly chelate both indium and yttrium. The structure of DTPA is shown below. Initially, DTPA, as a cyclic anhydride, was bound to antibody by an amide bond formed between one of DTPA's carboxylic acids and an amine on the antibody (Krejcarek, et al., Biochem. Biophys. Res. Commun. 77:581-585 (1976); Hnatowich, et al., Science 220:613-615 (1983)). This chelating structure was unstable *in vivo* with yttrium (Hnatowich, et al., J. Nucl. Med. 29:1428-1434 (1988)) because the loss of the carboxyl group decreased the number of donor ligands to 7. Substitutions to the carbon backbone of the molecule were developed as a means to attach the chelating group to the antibody without losing one of the ligands necessary for binding (Brechbiel, et al., Inorg. Chem. 25:2772-2781 (1986); Brechibiel, et al., Bioconjugate Chem. 2:187-194 (1991); Gansow, Nucl. Med. Biol. 18:369-381 (1991); Quadri, et al., Bioorg. Med. Chem. Lett. 2:1661-1664 (1992)). Bifunctional chelating agents attached to an antibody in this manner demonstrate stable chelation of yttrium *in vitro* and *in vivo* (Kozak, et al., Cancer Res. 49:2639-2644 (1989)). The backbone-substituted DTPA described herein, 2B3M-DTPA (Quadri, et al., 1992)), is shown below. The numbers indicate the position of the benzyl group (2B) and the methyl group (3M). The methyl group on carbon 3 provides additional stability to the coordination complex (Margerum, et al., Kinetics and mechanisms of complex formation and ligand exchange. In: Coordination Chemistry, monograph 174, Martell (ed) vol. 2 (American Chemical Society, Washington, D.C. 1978) pp. 1-220). The benzyl group attached to carbon 2 acts as a linker to join the chelating

group to the antibody through a thiourea bond created from the reaction of the isothiocyanato group (S=C=N) with an amino group from the antibody (Meares, et al., Anal. Biochem. 142:68-78 (1984)). The linker is stable *in vivo* and, depending on the application of the radioimmunoconjugate, may increase normal tissue toxicity. Linker-chelate structures that are more labile in normal tissue than tumor have been developed to decrease normal tissue toxicity (Deshpande, et al., Nucl. Med. Biol. 16:587-597 (1989); Quadri, et al., J. Nucl. Med. 34:938-945 (1993)).



DTPA



2B3M-DTPA

1

Chelating structures for radiometals.

The location of chelating groups on the immunoglobulin depends on the location and accessibility of the functional groups with which the reactive group on the linker can form a covalent bond. The isothiocyanato group reacts preferentially with the epsilon amino groups of lysine. The number of chelating groups attached to an antibody is controlled by the stoichiometric ratio between the chelating group and the antibody when they are reacted together. Low numbers of chelating groups are desirable, typically one group for IgG and four groups for IgM, in order to lessen the chance of blocking the antigen-binding sites and keep the specific activity, once the immunoconjugate is radiolabeled, within a reasonable range (10 mCi/mg for ^{90}Y). Higher activities increase radiolysis, i.e. breakage of the bond between the chelate and the protein by beta emissions.

Other bifunctional chelating structures have been developed including hinge-specific (Ali, et al., Bioconjug. Chem. 7:576-583 (1996)) and macrocyclic structures (Li, et al., Bioconjug. Chem. 4:275-283 (1993); Cox, et al., J. Chem. Soc. Perkin Trans. 1:2567-2576 (1990); Moi, et al., J. Am. Chem. Soc. 110:6266 (1988)). Hinge-specific bifunctional chelators are attached to antibodies through the sulfhydryl groups and form a single covalent bridge between two heavy chains in the hinge region. This produces immunoconjugates with exactly one chelate in a known location away from the antigen-binding site. Additionally, these chelating groups can stabilize immunoconjugate fragments by replacing the labile disulfide bonds holding the fragments together. Unmodified $F(ab')_2$ fragments are quickly reduced *in vivo* to Fab' fragments that are rapidly deposited in kidney (Quadri, et al., J. Nucl. Med. 34:2152-2159 (1993)). The macrocyclic chelates are cyclic polyamino-polycarboxylates as opposed to an open chain like DTPA. The chelation complexes formed with the macrocyclics have higher thermodynamic stability than those formed with DTPA (Harrison, et al., Nucl. Med. Biol. 18:469-476 (1991)), but they suffer from low specific activities, awkward labeling procedures and immunogenicity (Kosmas, et al., Cancer Res. 52:904-911 (1992)).

Methods and Dosages Required for *In vivo* Treatment

The targeted cytotoxic molecules can be administered intralesionally or intraregionally, so that the molecules remain in the targeted area for a time sufficient for binding to the tissue to be killed. Although described primarily with reference to tumors, there are many other types of tissue which it may be desirable to kill, such as the overproliferative tissue characteristic of endometriosis, or joint linings in the case of RA or other disorders characterized by immune complex deposition, inflammation, and overproliferation of tissue. As discussed above, the targeting molecule is designed to have two characteristics: selective binding to the targeted tissue, and large size, low diffusibility out of the region or tissue into which it is administered. Intralesional administration will typically be into the tissue to be killed, usually a solid tumor. Intraregional administration will be into a cavity such as the peritoneum or lungs. Types of cancer which can be treated include adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas (recurrent or persistent Hodgins' Disease; other than Hodgkin's lymphoma). Regions or compartments to be treated include intrapleural compartments, head and neck cancer, breast, ovary, peritoneum (peritoneal carcinomatosis), brain, prostrate, as well as other solid tumors and overproliferative tissue such as endometriosis in the uterus and peritoneum.

After selecting an appropriate targeted cytotoxic molecule, preferably an antigen-antibody-radioisotope combination, for the disease to be treated, administration variables must be considered, such as route of administration, administered activity, single or fractionated administration, protein dose, specific activity, predosing with unlabeled antibody. In a preferred embodiment, the administered activity level can be based on those used in previous studies or dosimetric calculations performed using the data acquired with the diagnostic radioisotope in the RIS phase of RIT. A radioimmunoconjugate can be administered once or divided into smaller activities over a short (1-2 week) period of time (i.e., fractionated). Fractionation can increase the therapeutic index by decreasing normal tissue toxicity (Schlom, et al., J. Natl. Cancer Inst. 82:763-771 (1990); Meredith, et

al., J. Nucl. Med. 33:1648-1653 (1992)). Fractionation does not appear to decrease the effects of RIT on tumor, unless a long fractionation schedule and a rapidly proliferating tumor are studied (Molthoff, et al., Int. J. Cancer 50:474-480 (1992)). The radiobiological principles of cell kill differ slightly for low, decreasing dose rate radiation via RIT and high dose rate external beam radiation. However, the surviving fractions of clonogenic malignant cells (and supposedly also clonogenic normal tissue cells) are similar after similar doses of radiation from RIT or external beam (Vriesendorp, et al., Radioimmunoglobulin therapy. In: High Dose Cancer Therapy. Armitage, et al. (eds) (Williams and Wilkins, Baltimore 1992) pp. 84-123).

High protein doses can activate complement, which will in turn produce side effects such as fevers, chills and shortness of breath. With high protein levels, tumors cells may be killed through immunologic mechanisms like complement-dependent tumor cytolysis or antibody-mediated cellular cytolysis. The analysis of RIT effects on tumor becomes more complicated if the tumor response is due to a combination of immunologic and radiation effects. Higher levels of protein are also more immunogenic. In general, it is advantageous for initial clinical studies to use a low protein dose (approximately 5 mg per administration).

The specific activity of a radioimmunoconjugate relates to its immunoreactivity, stability and therapeutic efficacy. If the specific activity is too high, the large number of chelates required per immunoglobulin molecule might reduce immunoreactivity. A high specific activity increases radiolysis. On the other hand, too low of a specific activity may result in undertreatment of a tumor since unlabeled immunoconjugates will compete with radiolabeled immunoconjugates for antigen binding. This is particularly important with an antigen that is expressed at a low density on a tumor cell.

Predosing with cold antibody has increased tumor deposition of radioactivity in some clinical trials using i.v. administration (DeNardo, et al. Cancer 73:1023-1032 (1994)). This may occur through the saturation of receptor sites that are involved in the metabolism of immunoglobulins or

through immunologic mechanisms by which the tumor becomes more accessible to the radioimmunoconjugate.

Two Stage Imaging/ Therapy

The demonstration of biodistribution and tumor targeting of a radioimmunoconjugate through gamma camera imaging is a major strength of RIT that is not available for other cancer treatment modalities. Imaging can be used to screen patients so that only those patients expected to benefit from therapy receive the immunoconjugate labeled with a high activity of the therapeutic radioisotope. However, the diagnostic part of RIT can be separated from the therapeutic part of RIT by employing the same immunoconjugate twice but radiolabeled with a different radioisotope for each administration. For the diagnostic administration, the immunoconjugate would be radiolabeled with a pure gamma-emitting radioisotope like indium-111 (^{111}In) or technetium-99m (^{99m}Tc). Both of these isotopes emit gamma rays within the appropriate energy range for imaging, (100-250 keV). Energies below this range are not penetrating enough to reach an external imaging device. Higher energy levels are difficult to collimate and provide diagnostic images with poor resolution. The short-half life of ^{99m}Tc restricts its use to immunoconjugates with rapid tumor uptake. The use of ^{111}In-labeled immunoconjugate has been proposed to predict the *in vivo* behavior of an immunoconjugate radiolabeled with ^{90}Y, a pure beta-emitter, since they have similar half-lives and comparable chelation chemistry (Vriesendorp, et al., Cancer. Res. (suppl) 55:5888s-5892s (1995); Vriesendorp, et al., Radioimmunoglobulin therapy. 1992); DeNardo, et al., J. Nucl. Med. 36:829-836 (1995); Leichner, et al., Int. J. Radiat. Oncol. Biol. Phys. 14:1033-1042 (1988)).

An additional advantage of using two separate radioisotopes, one for imaging and one for therapy, is that it allows for outpatient treatment. The low amount of radioactivity used diagnostically does not represent a radiation hazard, while the radiation emitted by a therapeutic pure beta-emitter will largely be absorbed in the vicinity of the tumor. This treatment scheme is dependent on similar pharmacokinetics for both radiolabeled

reagents and requires a stable means of attaching both radiometals to the antibody, which is discussed below.

Implicit in a treatment program that uses tumor targeting as a selection criterion for treatment, is the presence of measurable disease, i.e. tumor with a diameter greater than 1 cm. Tumors smaller than 1 cm are usually not measurable by CT, by MRI or with an ^{111}In-labeled radioimmunoconjugate. For measurable disease (greater than 1 cm), ^{90}Y, with its long-range beta emission, has the advantage of providing a higher and more homogenous tumor dose than beta-emitters with a shorter range (Leichner, et al., Med. Phys. 20:529-534 (1993)).

Detailed protocols are described below for intraperitoneal administration of IgM for the treatment of periotoneal carcinomatosis and intralesional administration of anti-ferritin IgM for treatment of Hodgkins' disease, Kaposi's sarcoma, and head and neck cancer.

As described in the examples, the protocols are written as a two stage approach with ^{111}In-labeled IgM used to demonstrate tumor targeting and to provide dosimetric information and, when warranted, followed by therapy with the ^{90}Y-labeled IgM. For some applications like Kaposi's sarcoma, the ^{111}In-labeled IgM may not be needed after initial testing.

***I.p. administration*:**

An indwelling catheter will be inserted transcutaneously. Free flow within the peritoneal cavity needs to be demonstrated with a radiographic contrast medium. If there are no serious hindrances to the dispersion of the contrast media, like adhesions, then the radioimmunoconjugate will be administered following the infusion of 1 to 1.5 l. of D5-1/4 NS (dextrose-saline solution).

For ^{111}In-labeled IgM, approximately 0.5 mg of IgM labeled with 1 mCi will be used.

For ^{90}Y-labeled IgM, the initial dose will be 1.0 mg of IgM labeled with 5 mCi. The next set of patients will be administered 1.0 mg of IgM labeled with 10 mCi. The last set of patients will be administered 2 x 1.0 mg of IgM labeled with 5 mCi. Other effective dosage ranges can be determined in the same manner using routine testing.

I.L administration:

Depending on the site and size of the tumor, one or more injections will be made.

For the ^{111}In-labeled IgM, approximately 0.5 mg of IgM labeled with 0.3 mCi of ^{111}In will be injected.

For the ^{90}Y-labeled IgM, the initial activity will be approximately 0.01 mg of IgM labeled with 0.1 mCi of ^{90}Y per 1 cc^3 of tumor volume. The next activity to be tested will be 0.2 mCi per 1 cc^3 of tumor volume. If this is well tolerated then the final activity will be 0.4 mCi per 1 cc^3 of tumor volume. Other effective dosage ranges can be determined in the same manner using routine testing.

Treatment of Disorders other than Tumors

Although described herein primarily with reference to treatment of tumors or other types of overproliferation of benign tissue, such as endometriosis, the conjugates can also be administered for treatment of disorders induced by deposition of immune complexes, such as in RA, or overproliferation of tissue.

RA is a chronic inflammatory arthropathy of unknown cause, but with a suspected autoimmune component in its pathogenesis. RA is a serious invalidating and life shortening disease with a major socio-economic impact due to its high incidence and prevalence throughout the Western World. It is most common in women over 60 years of age, but can occur even in adolescents. Curative therapy is not available at this time. Chronic palliative therapies are used with serious iatrogenic side effects. For the painful inflammation of larger joints surgical intervention (synoviectomy) is ineffective and has been abandoned in most Centers.

In RA, synovial membrane proliferation is induced by lymphokines provoked by autoreactive immune cells in the tissues surrounding the joint. Radiation of the immune cells decreases lymphokine production by inducing lethal DNA damage in these cells and interrupts the synovial proliferation. Swelling and pain decreases in the joint affected with RA. Intra-articular instillation with colloidal Y-90 has been applied mainly in Europe with a 50% response rate of approximately two years duration. (DJMcCarthy and

WJKoopman Arthritis and Allied Conditions 12th edition. 1993, Lea & Febiger, Philadelphia) A number of papers have reported on the efficacy of treatment of RA by radiation induced synovectomy in animal models and human clinical studies. These studies have used colloidal chromic phosphate 32P, colloidal 198Au, rhenium 186, dysprosium-165, 90Y-silicate (see, for example, Spooren, et al., Eur. J. Nucl. Med. 10(9-10):441-445 (1985); Boerbooms, et al., Eur. J. Nucl. Med. 10(9-10):446-449 (1985); Zuckerman, et al., Int. J. Rad. Appl. Instrum [B] 14(3):211-218 (1987); Aguilera, et al., Rev. Med. Chil. 122(11):1283-1288 (1994)).

As used herein in a preferred embodiment for treatment of RA, normal human IgM (which can be obtained, for example, from Centra processing blood products, that usually discard this protein for lack of clinical use) is labelled with Y-90 and Y-90 labeled IgM instillations are used in place of colloidal Y-90. The advantages of the IgM are that it is a soluble product leading to better diffusion in the joint fluids and better dose distribution for all synovial membranes. The size of the IgM prevents it from entering blood vessels and leaving the intra-articular space. Some radioactive IgM might translocate to lymph nodes draining the joint due to the insertion of IgM in lymphvessels through the fenestrations common to those vessels. This might be of some additional therapeutic benefit if these nodes contain radiosensitive autoreactive lymphocytes. Toxicity is not anticipated. Dosing would be based on the volume of the intra-articular fluids and be 0.1 mCi per cc for initial studies. The initial patient population for study is large joint RA patients, Brooker grades 2-3. In summary the application would provide a cheap, out patient, patient friendly RA treatment.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Preparation of Therapeutic Radiolabeled anti-tumor IgMs.

Monoclonal Antibodies

A human monoclonal antibody secreted by the AC6C3 heterohybridoma was used in these experiments (Freedman, et al.,

Hybridoma 10:21-33 (1991); Chen, et al., Hum. Antibodies Hybridomas 5:131-142 (1994)). It is of the IgM isotype and is reactive with the cell membranes of human ovarian, breast and colon carcinomas, and certain other malignancies. At approximately annual intervals, the hybridoma is subjected to recloning and the secreted antibody tested for reactivity against an ovarian tumor cell line (SKOV3, American Type Culture Collection, Rockville, MD) by fluorescence-activated cell sorter (FACS) analysis. The experiments presented here were conducted with the antibody secreted by the recloned cell line, AC6C3-2B12, hereafter referred to as 2B12. An isotype matched human monoclonal IgM (CH-1B9) was utilized as an irrelevant control.

The human monoclonal IgM CR4E8 (Chen, et al. Hum. Antibodies Hybridomas 5:131-142 (1994)) recognizes a 55 kDa cell surface membrane protein found on a human cervical carcinoma cell line (SW756). It is reactive with squamous cell carcinomas of both cervical and head and neck origin along with breast and colon malignancies. An isotype matched human monoclonal IgM (CH-1B9) served as a specificity control.

Antibody Production

The hybridomas were cultured in either tissue culture flasks or spin culture flasks in Iscove's Modified Dulbecco's Medium (IMDM) with 20% v/v fetal bovine serum (FBS), 10% NCTC 109 (Life Technologies, Gaithersburg, MD), insulin (5 μ g/ml) and transferrin (5 μ g/ml). After the cells were at a suitable density and good viability, the growth media was removed by centrifuging the cultures in sterile centrifuge bottles. The media was poured off and then the cells were resuspended in the production media and placed back into sterile culture flasks. The production media contains only IMDM and insulin (5 μg/ml) so it is devoid of serum immunoglobulins that copurify with the monoclonal antibodies. After 3-4 days, when the viability dropped to 20-40%, the cell culture supernate was harvested by centrifuging the culture in sterile 500 ml centrifuge tubes. The supernate was poured off and saved and then sterile filtered using a 0.22 m filter. Sodium azide, 0.005% w/v, was added to the supernate which was then stored at 4°C.

Antibody Purification

The supernate (1-3 L) was concentrated on a CH2RS spiral cartridge concentrator (Amicon, Beverly, MA) equipped with 1 ft^2, 30 K or 100 K MW cutoff membrane. The volume of liquid was concentrated down to less than 200 ml by running the apparatus at a pump speed of 7-8 and a back pressure of 25-30 psi. Next 1 L of 0.1 M PBS, pH 7.3, with 1 mM EDTA was added and the solution reconcentrated back down to less than 200 ml. This was repeated with 0.1 M PBS and then the concentrated supernatant solution was collected. The filtration apparatus was then washed twice with approximately 200 ml of 0.1 M PBS which was collected.

The concentrated supernatant solution was then further concentrated using a 8200 stirred cell (Amicon, Beverly, MA) equipped with a 100 K MW cutoff membrane and charged with 10-15 psi of nitrogen gas. The solution was reduced to approximately 30 ml and then the first wash from above was added. The solution was reconcentrated and then the second wash was added. The solution was reconcentrated to approximately 25 ml and then collected. The apparatus was washed with 10 ml of PBS which was then added to the concentrated solution.

The solution at this point has been concentrated approximately 100-fold. The metal ions were removed through chelation with the EDTA and most of the proteins and other molecules below 100 kDa in size were filtered out. Large proteins were still present which were then removed by size-exclusion chromatography using a 2.5 x 100 cm column packed with Sephacryl S-300 HR (Pharmacia Biotech, Piscataway, NJ). Approximately 5-6 ml of the concentrated antibody solution was loaded on the column and the antibodies were eluted with 0.1 M PBS containing 0.001% NaN_3. Fractions were collected every 70 seconds starting at 160 min after elution commenced.

The IgM fraction eluted first and could usually be visually identified. Conformation of the peak was obtained by measuring the absorbance of the fractions at 280 nm. The integrity and the purity of the IgM fractions were checked by size-exclusion HPLC using a Bio-Silect SEC 250-5 column (Bio-Rad, Hercules, CA) with 0.2 M sodium phosphate buffer, pH 7.2, as the mobile phase. Fractions with a single peak that had a retention time equal to

that of a known IgM standard were kept. The fractions from several column purification runs were combined and the material was reanalyzed by HPLC. The purified IgM was stored at 4°C.

Fluorescence-Activated Cell Sorter Analysis

The reactivity of the immunoglobulins with the cell surface of their respective cell lines used in these experiments was demonstrated by FACS. A human ovarian carcinoma cell line (SKOV3), was used for 2B12. These cells were cultured in L15 media containing 10%(v/v) fetal bovine serum (FBS) in the presence of 5% CO_2 at 37°C. A human head and neck squamous cell cervical carcinoma cell line, 886, was obtained from Dr. Walter Hittleman (The University of Texas M. D. Anderson Cancer Center) (Sacks, et al., Int. J. Cancer 44:926-933 (1989)) and used with CR4E8. These cells were cultured in 1:1 mixture of Iscove's modified Dulbecco's media and McCoy's 5A media (Life Technologies, Gaithersburg, MD) containing 10% v/v FBS in the presence of 5% CO_2 at 37°C.

Cells were detached from the tissue culture flasks with Versene and washed with phosphate buffered saline (PBS) pH 7.3 containing 2% FBS and 0.02% NaN_3 (w/v). They were then incubated with 100 µl (100 µg) of human IgM for 60 min at 4°C, washed, and then incubated in a 1:100 dilution of FITC-conjugated goat antihuman $F(ab')_2$ for 30 min at 4°C. The cells were then washed and resuspended in PBS with 2% FCS and 0.02% NaN_3 followed by fixation in 2.0% (v/v) paraformaldehyde. Cell binding was examined with an EPICS profile analyzer (Coulter, Hialeah, FL).

Preparation of Immunoconjugates

To a solution of IgM (1×10^{-5} mmol) in 0.05 M Hepes buffer, pH 8.6, was added isothiocyanatobenzyl-3-methyl-diethylenetriamine pentaacetic acid (ITC-2B3M-DTPA) (1×10^{-4} mmol). The solution was gently swirled to dissolve the ITC-2B3M-DTPA. Then triethylamine, 1.5 M, pH 8.6 was added to the solution to yield a final concentration of the amine in the range of 7.5-10 mmol. The solution was then incubated at 4°C for 12 h. The immunoconjugate was purified from unreacted ITC-2B3M-DTPA by filtration in a Centricon-30 (Amicon Corp., Beverly, MA) and washed with 0.1 M PBS, pH 7.3. Purity was ascertained by size-exclusion HPLC using a

Bio-Silect SEC 250-5 column with 0.2 M sodium phosphate buffer, pH 7.2, as the mobile phase. The average number of DTPA molecules per IgM was then determined (Quadri, et al., J. Nucl. Med. 34:938-945 (1993)).

Radiolabeling of IgM-2B3M-DTPA Conjugates

^{111}In-Labeled Immunoconjugates

The ^{111}In-labeled immunoconjugates were prepared by mixing equal volumes of 0.6 M sodium acetate buffer, pH 5.3, with 0.06 M sodium citrate buffer, pH 5.5, and the immunoconjugate solution (10 mg/ml). Typically volumes of 100 µl were used. Next pure $^{111}InCl_3$ solution (typically 5 µ l, 1 mCi; New England Nuclear, Boston, MA) was added to the buffered immunoconjugate, mixed well, and allowed to incubate for 40 min. The radioimmunoconjugates were challenged with excess chelator by incubating the labeling mixture with 0.01 M diethylenetriamine pentaacetic acid (DTPA), pH 6.5, for 10 min. The radiolabeled IgM was purified on a Sephadex G50 gel column (1.5 x 20 cm) using 0.1 M PBS as the elutant. Fractions were collected and assayed with a CRC-15R dose calibrator (Capintec, Ramsey, NJ). The degree of radiometal incorporation and the purity of the radioimmunoconjugate was assessed at each step by instant thin-layer chromatography (ITLC) with saline as the mobile phase and thin-layer chromatography (TLC) using a 1:1 ratio of methanol and 10% (w/v) ammonium acetate in water as the mobile phase. The strips were cut in half and counted in a Cobra II gamma counter (Packard Instrument Co., Meriden, CT).

^{90}Y-Labeled Immunoconjugates

The ^{90}Y-labeled radioimmunoconjugates were prepared by mixing equal volumes of 2.0 M sodium acetate, pH 6.0, with the immunoconjugate solution (10 mg/ml). The combined volumes ranged from 100-200 l for the biodistribution studies to 300-600 µl for the therapeutic studies. To this was added pure $^{90}YCl_3$ solution (Battelle Pacific Northwest Laboratories, Richland, WA). Volumes and activities ranged from 1-5 µl, 2.0-5.0 mCi for the biodistribution studies and 10-20 µ l, 20-50 mCi for the therapeutic studies. The solution was mixed well and allowed to incubate for 60 min. The reaction was quenched with a hundred-fold excess of chelator using 0.01

M DTPA, pH 6.5. After a 10 min incubation, the solution was loaded onto a Sephadex G100 gel column (1.5 x 50 cm) and the radioimmunoconjugate eluted with 0.1 M PBS, pH 7.3. The fractions were collected and assayed for radioactivity using a dose calibrator. Purity was assessed at each step by ITLC with saline as the mobile phase and by TLC using a 1:4 ratio of 30% ammonium hydroxide and ethanol as the mobile phase.

All purified radioimmunoconjugate solutions were sterile filtered and then diluted to the appropriate activities using sterile 0.1 M PBS, pH 7.3. The radioimmunoconjugates had greater than 95% purity and were used within 2 h of their preparation.

Example 2: Administration of Radioisotope-labeled Antibodies into Tumors to Determine Biodistribution.

Materials and Methods

Radiolabelled Antibodies

Radiolabelled anti-tumor antibodies were prepared as described in Example 1.

The purified antibodies demonstrated cell-surface reactivity by FACS of 86% for CR4E8 versus 12% for CH-1B9. Size-exclusion HPLC analysis demonstrated a purity of 98% for the immunoconjugates after derivatization. An average of four 2B3M-DTPA molecules were conjugated to each immunoglobulin. Moreover, after purification of the radioimmunoconjugates at least 96% of the radioactivity was antibody bound and more than 90% of the radioactivity remained bound when incubated for up to 72 h at 37°C in human serum.

Preparation of ^{90}Y-Aggregate

Five microliters of YCl_3 (3.3-7.7 mCi) was mixed with 50 μ l of 0.05 N NaOH. The solution was vortexed vigorously. One hundred microliters of a 12.5% solution of human serum albumin in PBS, pH 7.3, was added. The mixture was vortexed again and then allowed to incubate for 1 h. A gelatinous substance formed. An aliquot of the aggregate was resuspended with sterile 0.1 M PBS, pH 7.3, for injection into mice. The suspension was checked by ITLC and TLC to ensure that the presence of radioactivity was limited to the aggregate phase. The ^{90}Y-aggregate preparations were greater than 99% pure and were administered within 1 h of their preparation.

Tumor Inoculation

I.P. Model

A human epithelial ovarian carcinoma cell line, SKOV3-NMP2 (Mujoo, et al., Oncogene 12:1617-1623 (1996)), was obtained from Dr. Kalpana Mujoo (The University of Texas M. D. Anderson Cancer Center). It was grown in MEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C. Cells were detached with 0.25% trypsin at 37°C for 2 min. The trypsin was neutralized with media, and the cells were then centrifuged at 800 x g. The cell pellet was then resuspended in media at the required cell density. Female athymic nude mice, 6-10 weeks old, received 0.2 ml of the cell suspension through i.p. administration using a 30-gauge needle. For the biodistribution, therapy and tumor burden experiments, mice received 2.5 x 10^6 cells. For the study of the effect of cell number on survival, mice received either 5 x 10^4, 5 x 10^5, 2.5 x 10^6 or 10^7 cells.

I.L. Model

The human head and neck squamous cell carcinoma cell line, 886, was cultured as above. Cells were detached with 0.1 % trypsin at 37°C for 2 minutes. The trypsin was neutralized with media and the cells were centrifuged at 800 x g. The cell pellet was resuspended in media to a density of 8 x 10^7 cells per ml. Male athymic nude mice, 6-10 weeks old, were injected with 0.1 ml of cell suspension subcutaneously using a 30-gauge needle.

The mice were housed in filter-top cages and provided with sterile food and water. Animal studies were conducted in compliance with the USDA

and Animal Welfare Act. Animal protocols were approved by the Animal Care and Use Committee at the M.D. Anderson Cancer Center.

Biodistribution Studies

I.P. Model

The reagents were administered to mice that received the tumor cells inoculum 12 days earlier. Both the i.p. and i.v. treated mice received approximately 200 μ l of the radioimmunoconjugate. The ^{111}In-treated mice received 10-15 μCi, except for the mice treated for the measurement of whole-body retention of radioactivity. They received 55 μCi. The ^{90}Y-IgM-treated mice received 40 μCi. The ^{90}Y-aggregate was administered i.p. to the mice. They received 30 μCi in approximately 200 μ l.

Mice treated i.p. were euthanized at 3, 24, 48, and 96 h post injection. Mice treated i.v. were euthanized at 24 and 48 h posttreatment. Blood was drawn by cardiac puncture and weighed. Normal tissue and tumor nodules were excised, rinsed in PBS, blotted dry, weighed, and then counted in a gamma counter along with the blood samples. The results were corrected for radioactive decay and were expressed as percent injected dose per gram of tissue (% ID/g).

I.L. Model

The reagents were administered to mice inoculated with tumor cells 4-5 weeks earlier. Tumors sizes ranged from 8-12 mm in diameter at the time of administration. All the i.l. treated mice received a single injection into the center of their tumor with approximately 10-20 μl of the radioimmunoconjugate. The i.v. treated mice received approximately 200 μl of the radioimmunoconjugate. The ^{111}In-IgM treated mice received approximately 5 -10 μCi of activity and the ^{90}Y-IgM treated mice received approximately 20 μCi of activity. The ^{90}Y-aggregate treated mice received approximately 5-10 μl at an activity range of 10-20 μCi.

Mice treated i.l. with a radioimmunoconjugate were euthanized at 3, 24, 48, 96 and 144 h post injection. Mice treated by i.v. administration were euthanized at 24 and 48 h posttreatment. Mice treated i.l. with ^{90}Y-aggregate were sacrificed at 3, 24, 48 and 96 h post administration. Blood was drawn

by cardiac puncture and weighed. Normal tissue and tumors were excised, rinsed in PBS, blotted, weighed, then counted in a gamma counter along with the blood samples. The uptake of radioactivity was expressed as % ID/g.

Therapy Studies

Intraperitoneal Therapy

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier, received unlabeled immunoconjugate (10 μg) in 200 μ l volume of 0.1M PBS i.p.

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier, received graded activities, 50, 100, 200, 300, 400 and 500 μCi, of ^{90}Y-labeled 2B12 in a volume of 200 μl. Ten animals were used for each activity. An additional 5 animals received 200 μCi in a volume of 1 ml. Mice were screened after four hours for retention of radioactivity by placing them inside a 50 ml centrifuge tube which was then placed inside a dose calibrator. Mice were assigned to the dose ranges indicated by the calibrator.

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier received either 75 μCi x 2, weekly; 100 μCi x 2, separated by 3 weeks; 165 μCi x 2, weekly; 170 μCi x 3, weekly; or 200 μCi x 2, separated by 3 weeks. The volume of the injectate was 200 μl. Mice were screened after each administration in the dose calibrator to ensure that they retained the administered radioactivity.

Intralesional Therapy

Nude mice bearing s.c. tumors 8-12 mm in diameter received i.l. 10 μg of the unlabeled immunoconjugate in 10 μl volume of 0.1 M PBS.

Nude mice bearing s.c. tumors 5-12 mm in diameter received graded activities, 20-1000 μCi, of the radioimmunoconjugate in a volume of 5-20 μl. Mice were placed in a dose calibrator after administration to check the level of activity retained in the tumors.

Nude mice bearing s.c. tumors 8-11 mm in diameter were injected with 100-400 μCi of the ^{90}Y-aggregate in a volume of 5-10 μl.

Calculation of Tumor Size

Tumor size was checked every 3-4 days. Tumor volumes were calculated by the formula:

$$\text{Volume} = \pi/6 \times \text{length} \times \text{width} \times \text{height}$$

The % ID/g of tumors for both ^{111}In-labeled CR4E8 (tumor-reactive) and ^{111}In-labeled CH-1B9 (irrelevant) antibodies were obtained from the i.l. biodistribution experiments and plotted with the % ID/g as the abscissa and time as the ordinate. The area under the curve for both radioimmunoconjugates was obtained by summation of the area of the trapezoids on the graphs.

Since measurement of tumor response in the intraperitoneal model was not possible without either performing surgery or sacrificing the animals, an indirect way of measuring tumor response was sought. It was reasoned that the number of surviving tumor cells in the mice might be obtained by comparing their mean survival time (S_{50}) to the mean survival time of untreated mice that were inoculated with different numbers of tumor cells. The number of cells needed to produce a given S_{50} can be found. This number divided by the initial injected standard number of cells, 2.5×10^6 in the treated mice provided the surviving fraction (SF). This demonstrates a log-linear relationship between the surviving fraction of cells in the treated mice and the administered activity. This survival curve lacks a quadratic function that is typically observed after high doses of high dose rate, sparsely ionizing radiation. The model commonly used to describe radiation survival curves produced by sparsely ionizing radiation is the linear-quadratic model (Chadwick, et al., Phys. Med. Biol. 18:78-87 (1973)). With this model,

$$SF = e^{-[\alpha D + \beta D^2]}$$

where D = dose, $e^{-[\alpha D]}$ = cell death by the linear component and $e^{-[\beta D2]}$ = cell death produced by the quadratic component. The original hypothesis of this radiation survival curve model postulated that the component described a single ionization track that produced a double strand break in nuclear DNA. While the component described two ionization tracks that each produced a single strand break. Two "beta" breaks occuring close together in space and

time can form a double strand break in DNA. Only double strand DNA breaks are thought to correlate with the lethal effects of radiation on cells. The linear-quadratic model predicts that x-rays and gamma rays (high dose rate but short duration) employed clinically will be more effective than RIT which lacks the component. The low dose rate of RIT allows for repair of single strand breaks. However RIT optimizes other modes of cell killing, such as accumulation of tumor cells in the more radiosensitive G_2 phase of the cell cycle (Marin, et al., Int. J. Radiat. Oncol. Biol. Phys. 21:397-402 (1991); Knowx, et al., Radiat. Res. 135:24-31 (1992)), accelerated dose delivery or increased tumor cell kill by a process described as protracted exposure sensitization (PES) (Williams, et al. Int. J. Radiat. Oncol. Biol. Phys. 24, 699-704 (1992)). These additional mechanisms of tumor cell kill make up for some of the tumor cell kill lost by the low initial dose rate of RIT. Only small differences in surviving fractions between equivalent doses of RIT and external beam radiation have been seen in experimental animal models (Fowler, et al., Int. J. Radiat. Oncol. Biol. Pphys. 1261-1269 (1990); Fowler, et al., Int. J. Radiat. Oncol. Biol. Phys. 18:1261-1269 (1990; Knox, Cancer Res. 50:4935-4950 (1990); Neacy, et al. J. Nucl. Med. 27:902-903 (1986)).

A drawback of the peritoneal carcinomatosis model is that tumor volume at the time of administration of the radioimmunoconjugate is not accurately known. A direct calculation of dose (energy deposited in a given volume) is therefore not possible. Indirect estimates of tumor doses from RIT can be made by extrapolation of the observed biologic responses. In the section on surviving fraction of tumor cells, calculations are given for determining the number of surviving tumor cells after i.p. RIT. In this section, another model used to describe the shape of a radiation survival curve, the multi-target single hit model (Johns, et al., The Physics of Radiology. 4th ed. Charles C. Thomas (Springfield, IL 1983) pp.679-681) will be employed to simplify the calculations for estimating the absorbed radiation dose from i.p. RIT. In the multi-target model,

$$SF = 1-(1-e^{-d/d\,0})^n$$

where d = the initial slope of the survival curve, d_0 = the final slope, and n = the width of the shoulder of the curve (the extrapolation number). After the highest total amount of radioactivity administered i.p., tumor kill is estimated to be 3.5 logs. If external beam radiation and i.p. RIT show similar surviving fractions after similar doses of radiation and if one assumes an n of 3 and a D_0 of 2.0 Gy as radiation survival curve characteristics of SKOV3 NMP2:, the amount of external beam radiation needed in 2.0 Gy daily fractions to obtain 3.5 log tumor cell kill is approximately 52 Gy. However, the maximum tolerated dose to whole abdomen of human patients by external beam is less than 30 Gy. If the SKOV3 NMP2 cell line radiation survival curve parameters are chosen to reflect a more radiation sensitive tumor (n = 3, D_0 = 1.0 Gy), only 14 Gy of external beam radiation in 2 Gy fractions would be needed to reach an SF of 0.0005. Therefore, the current estimate for the dose delivered to tumor by RIT is between 3 and 10 Gy per 100 μCi of ^{90}Y (14-52 Gy divided by 500 μCi).

Autoradiography

Whole-Body

Two mice that had received i.p. ^{111}In-labeled 2B12 were sacrificed at 24 h postinjection. Their extremities were removed and the bodies frozen in 4% carboxymethyl cellulose. The frozen blocks were mounted on a cryomicrotome (Hacker Instruments, Fairfield, NJ), and sectioned into 50-m-thick coronal slices. Photographs were made of the sections, which were then mounted on tape and freeze dried. The sections were subsequently exposed to x-ray film (X-Omat AR, Kodak, Rochester, NY) for 48 h before development of images.

Tumor Autoradiography and Histology

Male nude mice bearing 8-12 mm s.c. tumors were injected with 5-10 μC of either ^{90}Y-labeled CR or ^{90}Y-aggregate in a volume of 5-10 μl. Mice were sacrificed at 3, 24, 48, 96 and 144 h. The tumors were excised, rinsed in PBS, blotted, covered with Tissue-Tek embedding medium (Miles Inc., Elkhart, IN) and then frozen at -20°C. The frozen blocks were later trimmed and mounted on cooled chucks in a refrigerated cryostat. Serial sections, 8

m thick, were taken approximately every 80 m through the entire tumor for the purpose of autoradiography. Adjacent sections, 4 m thick, were taken for histology. The sections were collected on slides. The histology specimens were stained with hematoxylin and eosin. The autoradiographic sections were mounted in sequence on stiff paper, covered with plastic film and then placed in a cassette with a phosphorous storage screen (Molecular Dynamics, Sunnyvale, CA). The screen was exposed for 1-2 days and then read on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Chords were drawn across the tumor images on the screen and counts were obtained along the length of the chords. Autoradiographs were obtained by placing slides on hyperfilm-ECL (Amersham, Arlington Heights, IL) for several hours to several days to expose the film.

Gamma Camera Imaging

Four mice that received i.l. ^{111}In-labeled CR4E8 were scanned at 24, 44, 68, 116 and 164 h postinjection. Five mice that received i.l. ^{111}In-labeled CH-1B9 were scanned at 24, 48, 96 and 144 h. Imaging was performed with a Starport gamma camera (General Electric Co., Milwaukee, WI) fitted with a medium energy collimator. The data was then transferred to a Triad gamma camera (Trionix, Cleveland OH). Regions of interest (ROI) were drawn over the tumors and counts were obtained for each time point. The counts per second were calculated and for the 2nd and subsequent time points, they were corrected for physical decay. The data was plotted as the percent remaining radioactivity in the ROI versus time. Biologic half-lives of the radioimmunoconjugates were then obtained from the graphs.

Results

Biodistribution of CR4E8 administered either i.l. or i.v.

The biodistribution data comparing i.l. and i.v. administration of ^{111}In-labeled CR4E8 showed that intralesional administration resulted in high tumor uptake at 24 h (108% ID/g) and the uptake remained elevated at 48 h (104% ID/g). The normal organs had low uptake, with kidney (3% ID/g) and liver (2% ID/g) being the highest at 24 h and both organs at 3% ID/g at 48 h. All other organs had <2% ID/g uptake at both time points. Blood radioactivity was <1% ID/g at both time points. In comparison, i.v.

administration resulted in very low tumor targeting (<1% ID/g) at both time points. Liver uptake was high (35% ID/g) and spleen uptake was moderate (≈ 14% ID/g) at both 24 and 48 h. Kidney had the next highest uptake (4% ID/g) at both time points. The blood clearance of radioactivity was nearly complete (1% ID/g) at 24 h.

Biodistribution data comparing the i.1. administration of CR4E8 labeled with ^{111}In or ^{90}Y showed that tumor deposition of radioactivity was high at 3 h (109% ID/g for ^{111}In and 102% ID/g for ^{90}Y, P=0.438) and remained elevated throughout the study with 56% ID/g for ^{111}In and 44% ID/g for ^{90}Y at 144 h (P=0.324). No statistically significant difference was found between the tumor deposition and retention of radioactivity for the radioimmunoconjugates. The normal organs demonstrated low uptake of radioactivity with both radiometals. Kidney (3% ID/g at 24 and 48 h for ^{111}In and 96 h for ^{90}Y) and liver (3% ID/g at 48 h for ^{111}In) had the highest uptake. Blood and bone radioactivity were <0.7% ID/g at all time points for both radiometals. Normal organs had similar levels of uptake of radioactivity for both radioimmunoconjugates at most time points with the exception of ^{111}In being >^{90}Y for kidney at 24 h (P=0.003) and ^{90}Y>^{111}In for femur at 96 h (P=0.005) and at 144 h (P=0.026).

Tumor deposition and retention of radioactivity for i.1.administered tumor-reactive and irrelevant IgM radiolabeled with ^{111}In shows that at 3 h tumor-reactive IgM had a higher deposition of radioactivity (109% ID/g) than irrelevant IgM (90% Id/g), but this was not statistically significant (P=0.324). At 144 h, irrelevant IgM showed moderate tumor retention (34% ID/g), while tumor-reactive IgM was retained more effectively (56% ID/g). This difference was statistically significant (P=0.021). The tumor uptake ratios of tumor-reactive to irrelevant IgM were 1.2 for 3 h, 1.3 for 24 h, 1.2 for 48 h, 1.7 for 96 h and 1.6 for 144 h. Normal organ uptake of irrelevant IgM was low with kidney having the highest value (2% ID/g) at 48 and 96 h. Blood radioactivity of irrelevant IgM was <1% ID/g at all time points.

Biodistribution data comparing the i.l. administration of ^{90}Y-labeled tumor-reactive IgM with ^{90}Y-aggregate show that tumor deposition and retention of radioactivity were higher throughout the course of the experiment with ^{90}Y-aggregate (126% ID/g at 3 h and 109% ID/g at 96 h) compared to ^{90}Y-labeled IgM (102% ID/g at 3 h and 77% ID/g at 96 h), but the sample size for ^{90}Y-aggregate was too small (n=3) to allow statistical analysis. With ^{90}Y-aggregate, bone had the highest uptake of radioactivity (3% ID/g) at 24 and 96 h, followed by kidney (1-2% ID/g). The other normal organs showed low uptake of radioactivity (<1% ID/g). Blood radioactivity was very low (<0.1% ID/g) at all time points.

Representative autoradiographs of sections from tumors treated with i.l. ^{90}Y-labeled CR4E8 or ^{90}Y-aggregate show that with ^{90}Y-labeled CR4E8 the majority of the radioactivity is localized at the injection site 3 h after administration. Additional radioactivity is apparent along the needle track and the periphery of the tumor. By 24 h, the radioactivity is widespread and remains so through the end of the experiment. In contrast, the ^{90}Y-aggregate remains localized at the injection site or along the needle track as discrete point sources of radioactivity for the entire experiment.

Activity levels along lines drawn across tumor images obtained at 48 h confirm that a greater volume of distribution of administered activity was achieved with the soluble radiolabeled IgM.

Images of a representative nude mouse injected 1.l. with ^{111}In-labeled tumor-reactive IgM show that the tumor deposition of radioactivity is high, with little uptake by the normal organs. The radioactivity remained localized and appears to become more homogenous within the tumor over time. The ROI analysis yielded tumor half-lives of 157 h for the tumor-reactive IgM and 96 h for the irrelevant IgM. Tumor half-lives determined by ROI analysis are similar to half-lives obtained in the biodistribution studies.

The whole-body retention of radioactivity in nude mice following the i.l. administration of ^{111}In-or ^{90}Y-labeled tumor-reactive IgM or ^{90}Y-aggregate shows that the elimination was monophasic for all reagents. Half-lives of approximately 73 h for ^{111}In-labeled IgM, 88 h for ^{90}Y-labeled IgM and 2200 h for ^{90}Y-aggregate were observed. The tumor half-lives of the

radioimmunoconjugates derived from the biodistribution experiments (143 h for ^{111}In-labeled IgM and 128 h for ^{90}Y-labeled IgM) were longer than the whole-body half-lives. In contrast, the tumor half-life of ^{90}Y-aggregate was shorter (475 h) than the whole-body half-life (2200 h).

Tumor Growth following i.l. Administration

When the unlabeled immunoconjugate was administered i.l., all mice showed continuous tumor growth like untreated mice and were euthanized when their tumors reached ≥15 mm in diameter. The "cold" immunoconjugate is not an effective immunotherapeutic agent. Metastasis (lymphatic or hematogenous) were not observed in this model.

When the radioimmunoconjugate was administered i.l, tumors were calculated to have received 80 Gy per 100 μCi of ^{90}Y-labeled IgM. The responses of animals treated with various activities of ^{90}Y-labeled CR4E8 is shown in Figure 1. All treated animals responded. As the majority of the tumors were rapidly ablated and tumor recurrences could not be linked to the original primary with absolute certainty, the results are expressed as the initial treatment volume and whether the tumor was ablated (complete response) or regressed (partial response). The graph demonstrates an apparent dose, but not dose/volume effect. Setting 150 μCi as an arbitrary cutoff point, the difference in tumor response above and below this level is statistical significance, $P = 0.001$. Tumor regression occurred with and without damage to the overlying skin. Tumor regression was marked by a color change in the tumor from pinkish white of viable tumor to a sallow, yellowish color.

Tumor ablation involved either crusting over and scabbing of the entire tumor or the tumor and surrounding normal skin were sloughed off resulting in an area of moist desquamation with a hole in the center where there once was tumor. Tumors frequently changed to a purplish pink color approximately 4-6 days after treatment prior to scabbing over. Other tumors turned whitish and necrosed from the center out. By 9-13 days, animals that received greater than 150 μCi to their tumor had a large area of moist desquamation that extended 2-4 mm beyond the margins of were the tumor was originally, as demonstrated by Figure 2. There were a few mice that had

extensive moist desquamation that appeared to result from leakage of the radioimmunoconjugate out of the tumor and into the surrounding subdermal tissue.

Fourteen out of 34 of the evaluable mice experienced tumor recurrence, as shown by Figure 3. Fewer mice are included in the tumor response duration analysis than in the analysis of tumor response and induction of moist desquamation due to the exclusion of animals with more than one single tumor nodule at the start of the experiment. This animal model relies on the subcutaneous inoculation of a cell suspension which may cause the formation of more than one tumor nodule in close proximity to each other. The biodistribution experiments demonstrated that both adjacent tumor masses and untreated masses on the opposite leg did not have significant uptake of radioactivity. Tumor recurrence were more frequent when less than 150 μCi was administered, $P = 0.039$. The majority of tumor recurrences occurred underneath the site of the original tumor. Tumor recurrence was less frequent in animals that showed moist desquamation.

When the ^{90}Y-Aggregate was administered i.l., all of the animals had a complete response. The morphologic changes that occurred in tumors after the ^{90}Y-aggregate treatment were markedly different from the changes in tumor after radioimmunoconjugate therapy. Tumors treated with high activities of ^{90}Y- aggregate were frequently intact at 9-14 days with central necrosis at the injection site. In contrast, tumors treated with the radioimmunoconjugate were sloughed off by this time point. Moist desquamation appeared later in the mice treated with ^{90}Y-aggregate and did not extend beyond the margin of the original palpable tumor.

Tumors recurred in 4 of the 8 mice treated with ^{90}Y-aggregate. In contrast, only 2 out of 12 of the mice treated with an equivalent activity of radiolabeled IgM had a tumor recurrence, $P = 0.06$.

Side Effects following i.l. Administration

No obvious, systemic toxicity was observed in any of the mice treated with ^{90}Y-labeled CR4E8, including mice that received 400-1000 μCi. Based on this fact and the very low level of radioactivity found in normal organs in the biodistribution studies, the weight of the mice was not measured. The

biodistribution data showed bone uptake and long whole-body retention of ^{90}Y in mice treated with ^{90}Y-aggregate, so their weight was monitored. They maintained their weight within 2% of their pretreatment weight signifying that no overt systemic toxicity was associated with this therapy.

Significant moist desquamation was observed in 13 out of 13 mice treated with > 150 μCi of the radioimmunoconjugate while mice treated with less than 150 μCi had moist desquamation in 6 out of 22 mice. This difference is statistically significant, $P = 0.00002$. Mice treated with the ^{90}Y-aggregate had moist desquamation of a reduced extent compared to mice treated with similar activities of the radioimmunoconjugate.

Blood counts in tumor-bearing mice following treatment with a single dose of ^{90}Y-labeled CR4E8 (average activity 308 μCi) were measured. On day 19 posttreatment, segmented neutrophils dropped to 40% and lymphocytes to 21% of their initial values. The RBC count was 92% of the initial value. On day 40 posttreatment, segmented neutrophils and lymphocytes were both at 58% of their initial value. The RBC count was 90% of the initial value.

Blood counts in tumor-bearing mice following treatment with a single dose of ^{90}Y-aggregate (average activity 287 μCi) were measured. On day 21 posttreatment, segmented neutrophils were at 71% and lymphocytes were at 40% of their initial values. The RBC count was stable at 103% of the initial value. On day 44 posttreatment, segmented neutrophils continued to drop to 51% of the initial value. Lymphocytes had recovered and were at 98% of the pretreatment value. The RBC count was at 101% of the initial value.

Mice treated with low activities (≤ 100 μCi) of the radioimmunoconjugate had mild skin damage in the form of reduced or absent adnexa (hair follicles, sebaceous and sweat glands). Some mice treated with higher activities had in addition to reduced or absent adnexa, fibrosis of the dermis, subcutis and skeletal muscle, vasculopathies and necrosis of skeletal muscle. The absence of skin adnexa is not indicative of radiation damage and is probably due to secondary healing of the denuded areas. The fibrosis and vasculopathies were attributed to radiation injury.

Tumor Growth Following Intraperitoneal Administration

The intraperitoneal administration of radioimmunoconjugate but not unlabeled immunoconjugate was able to prolong the lives of tumor bearing mice. In fact, there is a strong positive correlation between the amount of radioactivity administered and the prolongation of survival. For each 100 μCi of activity, from 0-300 μCi, the survival increased an average of 12 days. With high activities (300 μCi and above), early death occurred 12-14 days posttreatment.

Fractionated administration of radioimmunoconjugate also prolonged the lives of treated mice in a dose-responsive manner if the fractions were administered within an appropriate time period. For mice receiving a low activity (75-100 μCi) per fraction, the fractions could not be administered more than three weeks apart. With medium levels of activity (165-200 μCi) per fraction, the interval between fractions could be between 1-3 weeks. Mice treated in this manner demonstrated an increase in survival of 13 days for every 100 μCi of activity administered from 150 to 510 μCi. Fractionation increased the total activity that could be administered without producing weight loss or early death while at the same time maintaining the therapeutic efficacy.

High activities (300 μCi and above) administered as a single dose produced overt toxicity. Both weight loss in excess of 10% of initial body weight and early death were noted in groups treated at such doses. In contrast, fractionated administration did not cause overt toxicity even up to 510 Ci.

Blood counts were obtained from mice treated with an average of 133 Ci as a single dose and from mice that received 75 μCi x 2, separated by 2 weeks. Approximately 4 weeks from the initiation of treatment, both sets of mice demonstrated an increase in their segmented neutrophils. Mice treated with a single dose had more than twice as many segmented neutrophils than the fractionated mice. Lymphocytes in both groups of mice dropped to similar levels, 28% of the initial value.

The drop in peripheral blood lymphocyte count was expected as lymphocytes are known to be radiosensitive and to die rapidly from apoptosis after receiving low doses of radiation (Anderson, et al., Adv. Immunol. 24:215-335 (1976)). The increase in segmented neutrophils in the peripheral blood on the other hand was unexpected and may best be explained by the secretion of a growth factor specific for segmented neutrophils by the tumor cells or other cells in the peritoneal cavity. Indeed, various growth factors have been found in malignant human ascitic fluid (Hirte, et al., Proc. Ann. Meet. Am. Assoc. Cancer Res. 35:A258 (Abstract) (1994); Hirte, et al., Proc. Ann. Meet. Am. Assoc. Cancer Res. 34:A1428 (Abstract) (1993)), and growth factors specific for segmented neutrophils have been isolated and cloned: granulocyte-colony stimulating factor (G-CSF) (Nagata, et al., Nature 319:415-418 (1986)) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Wong, et al., Sciences 228:810-815 (1985)).

To confirm that the increase in segmented neutrophils was due to the tumor burden of the mice and not a compensatory mechanism in response to irradiation, mice from the tumor burden study had their blood sampled at different time points. There was an increase in the segmented neutrophil count over time. In one mouse that fortuitously did not have any tumor deposits, no increase in segmented neutrophils was observed.

The different responses noted for lymphocytes, granulocytes and erythrocytes after tumor inoculation and subsequent RIT indicate that the tumor has specific effects on the granulocytic line of hemopoiesis and not on hemopoietic stem cells. Similarly, i.p. RIT does not depress erythrocytes or lymphocytes to the same degree as granulocytes. Again, this indicates that decreased tumor burden leads to less granulocyte growth factor production and that relatively little stem cell damage was inflicted by i.p. RIT.

Discussion

The primary goal of this study was to test the concept of intracompartmental RIT with human monoclonal tumor-reactive IgM. This was accomplished through the use of 2 nude mouse models of human cancer and 2 locoregional modes of administration: i.p. and i.l. Both routes of

administration were compared against i.v. administration. Both the i.p. and i.l. routes demonstrated high, selective tumor deposition of radioactivity with lower normal tissue radioactivity than observed after i.v. administration.

When therapeutic activities were explored, i.p. administration was able to kill at least 3 logs of aggressively growing tumor without overt toxicity. Intralesional RIT produced complete tumor responses of long duration with minimal normal tissue toxicity. All therapeutic responses were due to radiation as the unlabeled "cold" immunoconjugate had no therapeutic effects.

Intralesional ^{90}Y-labeled CR4E8 was an effective therapeutic agent in a single administration. The only significant acute normal tissue side effect noticed was moist desquamation of the skin overlying and surrounding the tumor. Fractionation experiments to decrease this toxicity were not performed.

The ^{90}Y-aggregate remained localized in the center of the tumor nodule and was a less effective therapeutic agent. It also caused less moist desquamation presumably due to the larger distance between the radioisotope and the skin, leading to a lower radiation dose to skin.

This preclinical study demonstrates that i.l. but not i.v. administration of radiolabeled human tumor-reactive IgM results in high tumor radioactivity. Intralesionally administered radiolabeled IgM remained localized in the tumor, with low levels of radioactivity in the blood and subsequently low uptake of radioactivity in normal organs. In comparison, i.v. administered radiolabeled IgM was rapidly cleared from the blood with high liver and moderate spleen uptake. Intravenous administration of radiolabeled human IgM produced a similar biodistribution in other mouse models [Abeleve, Adv. Cancer Res. 14:295-350 (1971); Markoe, et al., Front Radiat. Ther. Oncol. 24:214-224 (1990) and may demonstrate significant liver uptake in human (Mach, et al. Cancer Res. 43:5593-5600 (1983)).

Intralesionally administered tumor-reactive IgM labeled with ^{111}In or ^{90}Y demonstrated similar high tumor uptake and retention. Normal tissue uptake of radioactivity was low, with kidney and liver having the highest uptakes (between 1 and 3% ID/g). Kidney activity may have been related

either to the metabolism of chelated light chains (Goldenberg, et al., J. Clin. Oncol. 9:548-564 (1991)) or to the urinary elimination of radioactive low-molecular-weight species; liver activity may have resulted from uptake of intact immunoglobulin from the blood.

Blood radioactivity was less than 0.7% ID/g for the tumor-reactive radioimmunoconjugates at all time points; this suggests that myelotoxicity will not be the dose-limiting normal tissue toxicity. The dose-limiting normal tissue, which remains to be defined, will probably be in the vicinity of the treated tumor. This tissue may vary, depending upon the location of the tumor and it may include vasculature, nervous, or interstitial tissue.

Bone radioactivity was less than 0.7% ID/g for the tumor-reactive radioimmunoconjugates at all time point. The clearance of radioactivity from bone was faster for ^{111}In-labeled IgM. This may account for the higher kidney activity seen for ^{111}In-labeled IgM at both 24 and 48 h. A small amount of ^{90}Y incorporated into bone could explain the slower bone clearance of ^{90}Y. The low level of bone radioactivity (0.5-0.6% ID/g) is not expected to have significant biologic effects (Lewis, et al., Hybridoma 14:115-120 (1995)).

The long biologic half-lives of the radioimmunoconjugates in tumor (4–6 days) indicates that greater effective half-lives of the radioimmunoconjugates and hence radiation doses could be achieved through the use of therapeutic radioisotopes with half-lives longer than ^{90}Y (2.7 days). With such isotopes, the observed difference in tumor retention between tumor-reactive and irrelevant IgM, which became statistically significant at 144 h, would be of greater therapeutic significance.

The i.1. administration of ^{90}Y-aggregate produced high tumor deposition of radioactivity which remained elevated longer than ^{90}Y-labeled tumor-reactive IgM and resulted in a biologic half-life of 20 days in tumor. While this longer half-life suggests that higher radiation doses to tumor could be administered with ^{90}Y-aggregate, comparison of whole-body half-lives with tumor half-lives and examination of tumor autoradiographs predict that overall 90Y-labeled IgM will be the superior therapeutic agent.

The different tumor and whole body half-lives of the radioimmunoconjugates compared to the ^{90}Y-aggregate suggest fundamental differences in the metabolism of these reagents. The tumor half-lives of the radioimmunoconjugates are longer than their whole-body half-lives. Radioactive fragments produced from the metabolism of the radioimmunoconjugates appear to be rapidly eliminated from the body. This is in contrast to what is observed with the ^{90}Y-aggregate where free ^{90}Y from ^{90}Y-aggregate appears to be retained in the body by transchelation to the bone matrix.

Autoradiographs from sectional tumors demonstrate that tumor-reactive IgM diffuses throughout the tumor, whereas ^{90}Y-aggregate remains localized at the injection site. Analysis of the distribution of the counts per pixel across lines drawn on the tumor images confirmed the greater distribution of radioactivity with the radioimmunoconjugate. Thus, the radioimmunoconjugate provides a more uniform dose distribution within the tumor than the ^{90}Y-aggregate. The combined advantages of more uniform dose distribution within the tumor and rapid elimination of metabolized radioimmunoconjugate should provide a therapeutic advantage to the radioimmunoconjugate.

Serial gamma camera imaging corroborated the results of the biodistribution studies. The images show that high initial tumor deposition of radioactivity is achieved with i.l. administration of radiolabeled IgM and that the radioactivity remains localized. Furthermore, the activity appeared to become more homogenous over time. Very little normal tissue uptake of radioactivity was evident with either tumor-reactive or irrelevant radiolabeled IgM. Tumor half-lives calculated by ROI analysis agree with those obtained in biodistribution experiments. Together, these results suggest that the serial imaging of a small group of animals with a gamma camera can replace the more animal-intensive biodistribution experiments in the initial screening of radioimmunoconjugates for local administration.

Example 3: Protocol for Intraperitoneal IgM for the treatment of perit neal carcinomat sis.

Patients with peritoneal carcinomatosis in general have one of the four primaries mentioned in this study: ovary, colon, pancreas, breast. Currently available treatment is unsatisfactory and median survival time is approximately three months.

Abstract

Protocol:

Patient Eligibility:

1. Histologically or cytologically confirmed epithelial ovarian carcinoma with extra ovarian peritoneal involvement - colon, breast, or pancreatic cancer in the peritoneal cavity.
2. Measurable disease.
3. Absence of small bowel obstruction.
4. Zubrod 0, 1, 2. Age ≥18 years. Negative pregnancy test.
5. Signed informed consent.

Treatment Plan:

1. Place temporary intraperitoneal catheter.
2. Verify free flow by radiographic contrast.
3. Instill 1-1.5 LD5 - 1/4 NS.
4. Obtain peritoneal fluid, blood and urine samples for immunoperoxidase staining and radiopharmacokinetic baseline studies.
5. Administer 1 mCi ^{111}In labeled AC6C3-H8 IP.
6. Sample peritoneal fluid, blood, urine 2, 20, 40, 72, and 150 hrs.
7. Whole body gamma scans at 2, 20, 40, 72 and 150 hrs.
8. SPECT at 41 hours of the abdomen.
9. Administer 5, 10 or 3x5 mCi of ^{90}Y labeled AC6C3-H8 IP after repeating steps 1 59 3.

Patient Evaluation:

1. Complete history and physical.
2. CBC with differential, platelets, PT/PTT.

3. Liver functions to include SGOT, SGPT, alkaline phosphatase and bilirubin.
4. Renal function tests including BUN, creatinine and urinalysis.
5. Abdominal and pelvic CT±contrast, chest x-ray and any other tests needed for tumor documentation.
6. 5cc serum for immunologic studies.
7. Review of all hospital records and pathology reports. Pathologic reviews of available specimens to confirm the diagnosis.

Statistical Considerations:

The main objectives of this study are to establish safety and to obtain information on potential utility. Data to be obtained from this study may be used in the design of future trails on a larger number of patients. The pilot trial will be stopped if tumor localization is not shown in the first 6 patients or if grade III or higher toxicity occurs in equal or more than one-third of patients. The first 6 patients will receive 5 mCi of ^{90}Y, the next 6 patients will receive 10 mCi of ^{90}Y, the last 6 patients will receive 5 mCi of ^{90}Y 3 times at 1 week intervals.

Reagents:

Antibodies

AC6C3-H8 is a new human tumor cell surface reactive monoclonal antibody (HMAB) obtained by fusing lymph node lymphocytes from a patient with epithelial ovarian carcinoma (EOC) with cells of the SPAZ 4 heterohybridoma line (Freedman, et al., Hybridoma 10:21-33 (1991)). This antibody is an IgM2 and recognizes a determinant expressed on the cell surface of an ovarian carcinoma cell line that can be detected by fluorescent activated cell sorter analysis (FACS) (Quadri, et al., J. Nucl. Med. 34:938-945 (1993)). The AC6C3 does not stain nylon wool purified nonadherent peripheral blood lymphocytes or red blood cells from normal donors. AC6C3 stains nonpermeabilized tissue sections of ovarian carcinoma and ovarian tumor xenografts using indirect immunoperoxidase. Most normal tissues including brain, liver, heart and kidney give negative or weak reactions to AC6C3. The antibody also reacts with other adenocarcinomas including breast and colon and may therefore be described as "pan-reactive".

The antibody mediates complement-dependent cytotoxicity and precipitates a 32Kd band by Western blotting immonoprecipitation experiments conducted on $SKOV_3$ cultured ovarian cancer cells. The association constant for AC6C3 is $2.3 \times 10^{10} M^{-1}$ which was determined by saturation binding studies (Quadri, et al. 1993). AC6C3 may have certain advantages in clinical trials in patients with peritoneal carcinomatosis: (i) The human mAb may be used in repeated dosing schedules assuming anti-human antibodies are not produced. (ii) Although IgG mAbs may be preferred for clinical trials, IgMs may also be attractive for intracavitary treatments because of their large size and likelihood of a prolonged residence time. Prolonged residence time of the mAb in the peritoneal cavity may promote greater concentrations of the mAb on the tumor cells. AC6C3-H8 is a novel entity with a potential role for in vivo diagnosis and therapy of ovarian carcinoma.

Purification of Human Monoclonal IgM Antibody (HuMoAb) from Culture Media:

The media supernatant was concentrated by using Amicon Stirred Cell unit mounted with PM30 membrane at 4°C in a cold room. The retentate containing IgM was purified on Sephacryl S-300 column (2.5 x 50cm) to isolate the pure IgM fraction. The column was eluted with 0.05 M PBS pH 7.4 at a flow rate of 1.5 ml per minute. Purity of IgM was analyze by size-exclusion HPLC using two gel filtration columns (GF 250 & GF 450; 25 x 0.94 cm i.d.) connected in tandem, and SDS-PAGE techniques. Protein concentration was measured by UV spectrophotometer at 280nm absorbence. The IgM protein was sterile filtered through 0.2 μm Acrodisc and stored in aliquots of 4.0 mg/ml in PBS at 4°C in sterile tubes.

Conjugation of Antibodies

Human IgM AC6C3-H8 was purified and conjugated with an average of two ITCB-DTPA chelating agents per IgM molecule (Leichner, et al., Antibody Immunoconjugates and Radiopharmaceuticals 2:125-144 (1989)). The purified immunoconjugates showed no loss of activity to the ovarian carcinoma cell line ($SKOV_3$) *in vitro*. The ^{111}In labeled immunoconjugates were injected intravenously into nude mice bearing a human ovarian carcinoma ($SKOV_3$) xenograft. The human IgM conjugate cleared rapidly

from the circulation with a T1/2 (half-life) in the blood of less than 10 hours. Most of the activity was retained in the liver because of the large molecular size of the IgM molecule. The tumor targeting peaked at 48 hours. the tumor-to-muscle and tumor-to-blood ratios were 4:1 and 2:1 respectively at day 6. The immunoscintigraphy study demonstrated that radioactivity peaked throughout the tumor mass at 2 days post IgM administration. The tumor uptake in the biodistribution study showed 4-6% injected dose/gram of tumor at 48 hrs (n=3). Direct intraperitoneal administration of AC6C3-H8 to nude mice with higher tumor radioactivity (60% of injected dose/g of tumor) - therapy with Y^{90} labeled IgM prolonged survival of mice with extensive peritoneal carcinomatosis three-fold. In mice, fractionated therapy (once per week) is more effective and less toxic than single fraction therapy.

Preparation of Immunoconjugate:

The chelator, isothiocyanatobenzyl-DTPA derivative (ITCB-DTPA), is synthesized by previously published techniques. ITCB-DTPA was reacted with amino group of lysine residue of antibody to conjugate through a thiourea linkage. Antibody (9.0 mg, 1×10^{-5} mmole) in 0.05 M Hepes buffer pH 7.6 was reacted with freshly prepared 100 µl aqueous solution of ITCB-DTPA (60 µg, 1×10^{-4} mmole) at a molar ratio of 1:10. The pH of the reaction mixture was adjusted to 7.6 by 0.2 m $NaHCO_3$ solution and mixture was incubated for two hr at room temperature. Two DTPA ligands were conjugated per antibody molecule. Prior to radiolabeling, MoAb-thiourea-DTPA conjugate was purified from unconjugated DTPA by dialysis against PBS.

Radiolabeling of Immunoconjugate:

An aliquot (100 µl) of pure $^{111}In\ Cl_3$ (8.0 mCi) in 0.05 N HCl is equilibrated with 100 l of 0.5 M acetate buffer pH 5.3 and 100 µl of 0.05 M citrate buffer pH 5.5. Two hundred microliters of immunoconjugate (2.0) mg) solution in PBS is added into buffered indium, mixed well, and incubated at room temperature for 30 min. The labeled immunoconjugate is separated from low molecular weight compounds by Sephadex G50 gel column (1.5 x 20 cm) chromatography using 0.05 M PBS as an eluent. The labeled immunoconjugage is collected and assayed in a dose calibrator, and

the labeling efficiency is determined by ITLC. The percentage radioactivity bound to the antibody is determined by TLC and HPLC analysis. The procedure for ^{90}Y Cl_3 labeling is similar, with the exception of different buffer with slightly higher pH and a G100 column instead of a G-50.

Quality Control Analysis:

Instant Thin Layer Chromatography (ITLC)

Radiolabeling yield of immunoconjugate is determined by ITLC using saline as developing solvent. Radioimmunoconjugate remains at origin while labeled DTPA moves with the solvent front. The ITLC strip is cut into three portions and counted on a gamma counter.

Thin Layer Chromatography (TLC)

Silica-gel TLC strip (1 x 12) is spotted with radiolabeled conjugate and developed by using methanol:10% NH_4OAc (1:1) as a mobile phase. In this TLC analysis, radiolabeled antibody appeared at R_f=0 and indium labeled DTPA/citrate at R_f=1.0. TLC strips are monitored by a radio chromatography scanner. the percent activity bound to the antibody fraction is determined by counting TLC strip in a gamma counter.

HPLC Analysis

Size exclusion HPLC analysis with a Bio-Sil SEC 250 column (300 x 7.8 mm i.d.) is used to determined the molecular weight and purity of radioimmunoconjugates.

The column was eluted with buffer (containing 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.9) at a flow rate of 1 ml/min.

Sterility and LAL Assay

The radioimmunoconjugate in PBS solution is filtered through 0.2 micron Acrodisc filter and tested for lack of pyrogens by Limulus Amebocyte Lysate assay (LAL). A small aliquot of the antibody batch (0.25 ml) is diluted to a final volume of 5 ml with 0.9% sodium chloride and tested for sterility using the protocol outlined by the FDA Code of Federal Regulation CFR 21, Part 210. Two ml aliquots are inoculated into fluid thioglycollate medium and soybean casein digest. The sample is incubated at 30-35°C, respectively for a period of 14 days. The aliquots are checked visually for bacterial growth. The pyrogen test is performed before the

administration of radiolabeled antibody, whereas the sterility test is completed for the whole batch only, not for individual patient administration.

Drug Composition

Indium-111 labeled human monoclonal IgM-DTPA conjugate in 5.0 ml PBS solution. The drug contains the following components:

Human Monoclonal IgM-DTPA	2.0 mg
Chelated Indium-111	5.0 mCi
0.05 M Phosphate Buffer Saline	5.0 ml
Human Serum Albumin (0.1%)	5.0 mg

Patients:

Patient Elgibility

Histologically or cytologically confirmed epithelial ovarian carcinoma, colon, breast or pancreatic carcinoma.

Patients with total or subtotal small bowel obstruction are not eligible.

Documentation of peritoneal carcinomatosis by clinical documentation, prior surgery, computerized tomography (CT) or magnetic resonance imaging (MRI).

Karnofsky ≥60, ECOG 0, 1 or 2.

Age ≥18 years.

Negative pregnancy test in women at risk.

No untreated active infection

Absolute granulocytes above 1000/mm^3 and platelets above 50,000/mm^3.

Pre-study Evaluation

Complete history and physical including height and weight documentation of signs and symptoms and performance status will be done in the week before the study commences (Appendix B).

Laboratory and Radiologic Studies

CBC with differential, platelets, PT/PTT.

Liver functions to include SGPT, alkaline phosphatase and bilirubin.

Renal function tests including BUN, creatinine and urinalysis.

Abdominal and pelvic CT ± contrast, chest x-ray and any other tests needed for tumor documentation.

5 cc serum as baseline for future F/U studies.

Review of all hospital records and pathology reports. Pathologic reviews of available specimens in studies to confirm diagnosis.

Evaluation During Study

Pharmacokinetics, tumor targeting and associations of uptake with clinical or radiological findings will be done as described below.

Blood, peritoneal fluid, urine sample, whole body gamma camera scan at 2 hours. Patient samples obtained and sampled under radiation safety precautions in Radiotherapy. Scan to be performed in Nuclear Medicine.

Repeat studies described in 6.3.1 at 20,40,72 and 150 hours. Scans will for peritoneum, and blood half-life by region of interest (ROI) analysis. Tissue distribution will be evaluated on scans as well. Inhomogeneities in radioimmunoconjugate distribution will be evaluated on SPECT (at 40 hours). Comparisons will be made with tumor deposits observed on CT. Tumor dosimetry will be done following the University of Nebraska protocol (Drs. Leichner, Hawkins, Akabani) (Hawkins, et al. IEEE Trans. Med. Imag. 7:135-148 (1988)) as soon as the required software is operational in the Arlington Cancer Center.

Recent radiologic findings (within 4 weeks) will be correlated with results of scanning.

Treatment:

Discussion of protocol and signed informed consent before entry.

A temporary intraperitoneal catheter will be inserted transcutaneously.

Verify free flow by injecting approximately 50 cc radiographic contrast IP. Under fluoroscopy orthogonal x-rays will be taken for documentation.

Infuse 1-1.5L D5-1/4 NS warmed to 37°C IP.

One hundred cc fluid removed for immunoperoxidase staining of tumor cells with biotinylated peroxidase conjugated HuMoAb and irrelevant HuMoAb and baseline for studies listed under t.6.

Administer 1 mCi ^{111}In labeled AC6C3-H8 IP in approximately 10cc. The antibody will be administered under the supervision of a physician.

Vital signs q 15 minutes for 2 hours. Patient in decubitis, anti-decubitis, left lateral and right lateral positions, prone and supine positions for 20 minutes each.

Five, ten or 3x five mCi of ^{90}Y labeled AC6C3-H8 will be administered following identical methods as described for the 111In labeled product. ^{90}Y labeled IgM will only be administered if the ^{111}In labeled IgM stays confined to the peritoneal cavity ()75% of injected dose) and tumor targeting is observed.

Dose of MoAb:

Starting dose will be 0.5 mg. If no, or poor, imaging is observed in first six patients next dose level will be 5 mg.

Evaluation and Management of Toxicity:

If 2 or more of the first 6 patients in either group develop grade III or IV toxicity, the protocol will be stopped. CBC and SMAC will be repeated weekly for the first 8 weeks after each administration and once a month thereafter, scans will be repeated at 2-month intervals.

Study End-Points:

Pharmacokinetics:

Compartmental and noncompartmental pharmacokinetic techniques will be used to determine the time area under the concentration curve, peak concentration, time to peak concentration, mean residence time distribution volume, clearance, intraperitoneal labeled antibody concentrations.

Immunotargeting:

Uptake and elimination of the radioimmunoconjugate will be studied for: (1) peritoneal cavity; (2) tumor nodules; (3) blood; (4) liver and spleen; (5) kidney; (6) urine. *In vivo* decay of radioactivity can be determined by planar views of the gamma camera. Tumor volumes and radioactive content of tumors and smaller volume normal tissues will require SPECT. The ideal radioimmunoconjugate will target tumor, have a long residence time in the peritoneal cavity with little or no spill-over into the blood. Normal tissue uptake should be low. The data points to be obtained will allow for

calculating the relative biodistribution of the radioimmunoconjugate over time in the listed compartments.

Tumor Dosimetry

Cumulative radioactivity in tumor volumes will be calculated by area under the curve techniques. Translation/extrapolation from Indium radioactivity to Yttrium dosimetry will be made for the planned administration of 5, 10 or 3 x 5 mCi of Yttrium labeled radioimmunoconjugate per kilogram, retrospectively when the appropriate computer programs have been installed and verified.

Criteria for Removal from Study:

Noncompliance or inability to complete follow-up studies. Grade III toxicity or tumor progression.

Example 4: Protocol for Intralesional Radiolabeled Antiferritin IgM of recurrent or persistent Hodgkin's Disease, Kaposi Sarcoma, and Head & Neck Cancer.

Approximately 7000 new cases of Hodgkin's disease (HD) are diagnosed each year in the United States. Initial treatment with chemotherapy, radiation or a combination of the two is successful in inducing a response in the vast majority of patients. Nonetheless approximately 30-40% of patients will relapse or fail to achieve an initial remission. Second line treatment with salvage chemotherapy is curative in a small group of patients. Second or third line treatment with high dose chemotherapy followed by autologous bone marrow transplantation may be curative in certain patients. Patients who fail second or "higher" line treatment and those who are not eligible for bone marrow transplantation are rarely cured and are candidates for Phase I studies. Phase I-II studies with ^{90}Y labeled polyclonal antiferritin have been completed. It was found to be an active single agent for relapsed Hodgkin's Disease with response rate of approximately 65% in a heavily pretreated poor prognosis group of patients. In a proportion of patients a single large mass (e.g. mediastinum) is a persistent problem after extensive prior chemo/radiation therapy.

Kaposi's Sarcoma (KS) is a vascular tumor arising from the mid-dermis. It consists of interweaving bands of spindle cells and irregular slit-

like vascular channels embedded in reticular and collagen fibers, infiltrations with mononuclear cells, and plasma cells. It was first described by the Austrian dermatologist Kaposi. Kaposi's Sarcoma (KS) occurs frequently in immunodeficient patients, such as HIV positive patients or allograft recipients. The skin, oral mucosa, lymph nodes and visceral organs such as the submucosa of the gastrointestinal tract, lung, liver and spleen may be involved. Systemic therapy for Kaposi's Sarcoma is poorly tolerated due to preexisting immunodeficiency or myelo-suppression. KS tumors are considered radiosensitive and palliation can be obtained with doses as low as 16 Gy, External beam radiation can be toxic in KS patients, especially in terms of severe radiation induced mucositis. All tested KS lesions contain ferritin (N=40).

The combination of tobacco and alcohol use are the best established and most significant carcinogens of mucosal malignancies in the head and neck area. The majority of patients have squamous cell carcinoma (HNSCCa) and present with large (> 6 cm) primaries and/or large lymph nodes metastases. Combination of radical surgery and high dose external beam radiation can control the disease in a proportion of the patients. However disease recurs in at least 30% of patients, who then become candidates for phase I chemotherapy. Distant metastases are relatively uncommon in such patients and the use of intralesional IgM could provide a new treatment option for such patients after prior high dose radiation. Up to 50% of squamous cell carcinoma's of the Head and Neck appear to contain significant amounts of ferritin.

Abstract

Protocol:

Patients:

Histological proof of recurrent or persistent Hodgkin's Disease, Kaposi Sarcoma, and Head & Neck Cancer.

Patients must be >10 years of age.

Patients must have measurable disease.

Treatment Plan:

0.3 mCi of indium-111 labeled antiferritin is deposited in the tumor mass. Whole body gamma camera imaging will be performed at 1, 20, 40, 120, and 165 hours after administration. SPECT scans will be performed of tumor containing areas at 40 and 165 hours after administration. Blood and urine samples will be taken at the same time points as for whole body gamma camera imaging. All urine will be collected for the first 48 hour after administration.

Patients will receive 0.1 mCi per gram of tumor of yttrium-90 labeled antiferritin which will be divided in three equal volume (0.5 cc) directly into three different parts of the tumor (intralesionaly). The yttrium-90 labeled antiferritin will only be given to the patients if the studies after indium-111 administration show that 90 percent or more of the deposited radioimmunoconjugates remains localized in tumor and draining lymph nodes. Blood and urine sample will be collected at 1, 20, 40, 120 and 165 hours after the yttrium-90 administration. The first 48 hours urine will also be collected. Bremsstrahlung scans will be performed at the same time points.

Procedures will be repeated twice with a week interval, and patients will receive a total activity of 0.3 mCi of yttrium-90 per gram of the tumor in three treatments over two weeks.

If five patients per histological tumor type have tolerated the procedure without acute side effects, the yttrium-90 activity will be escalated to 0.2 mCi per gram of tumor. If an additional five patients do not show acute side effects at the 0.2 mCi per gram of tumor level, an additional five patients will be done at the 0.4 mCi per gram of tumor level. Patients with Kaposi's Sarcoma will not receive more than 0.2 mCi of yttrium-90 per gram of tumor.

Statistical Considerations:

The study will be stopped if two out of five patients develop grade 3-4 acute side effects at any yttrium-90 level. If this happens for only one or two of the three tumor histologies under study, the study will continue in the uneffected tumor type(s).

Reagents:

The radioimmunoconjugate is prepared in four consecutive phases: Preparation of antiferritin antibody; Synthesis of immunoconjugate; Radiolabeling of immunoconjugate; Quality control of radioimmunoconjugate prior to clinical administration.

Preparation of Monoclonal Antibody:

Human ferritin isolated from a spleen of a patient with Hodgkin's disease was injected repeatedly with adjuvant in mice for monoclonal antibody production. After fusion, clones were screened. MDA 101 produced high affinity antiferritin. The clone was adjusted to hollow fiber culture and a batch of IgM was produced and purified. Antibodies were dispensed in single patient vials and checked for immunoreactivity activity (against ferritin), sterility and pyrogenicity.

Synthesis of immunoconjugate:

The isothiocyanatobenzyl - DTPA chelate is synthesized as described above.

Radiolabeling of the immunoconjugates:

The chelate conjugated antibody is maintained at 4°C until used. Radiolabeling is accomplished by adding to an (2 mg/ml) aliquot of immunoconjugate at room temperature purified carrier-free, freshly prepared $^{[1]}InCl_3$ that is dissolved in a mixture of acetate and citrate buffers at pH 5.5. Chelation of $^{[1]}In$ occurs within 30 minutes. The chelate is challenged by free DTPA in greater than 100 fold excess. The indium labeled immunoconjugates are purified on Sephadex G50 gel column chromatography (1.5 X 20 cm) using 0.05 M phosphate buffered saline. ^{90}Y radiolabeling is achieved by using a acetate buffer (2.0 M), pH 6.0 at room temperature similar to the $^{[1]}In$ chelation. ^{90}Y labeled immunoconjugates are purified on Sephadex G-100 column (1.5 X 30 cm) chromatography and eluted with 0.05 M PBS.

Quality Control Analysis of Radioimmunoconjugates:

The radioimmunoconjugate is filtered through 0.22 micron sterile filter and tested for lack of pyrogens by a Limulus Amebocyte Lysate (LAL) assay.

The purified antibody is tested for the presence of unconjugated $^{[1]}$In-DTPA by thin layer chromatography using a solvent mixture containing 10% ammonium acetate and methanol in 1 : 1 ratio (TLC silica) or 0.9% saline (ITLC silica). These solvent systems leave the labeled antibody at the origin and unconjugated $^{[1]}$In-DTPA at R_f1.0. The TLC and ITLC are completed before the administration of the antibody.

The radiolabeled immunoconjugates are also analyzed by size-exclusion HPLC to determine any colloid formation, and tested for radiochemical purity. The integrity of the radiolabeled antibody, prior to administration, is examined using HPLC. Analyses are performed using size-exclusion filtration Bio-silect SEC 250-5 (0.78 X 30 cm) column equilibrated in 100 mM sodium phosphate containing 150 mM sodium chloride, pH 6.8. Antibody samples (25 µl) are applied and the column runs at a flow rate 1.0 ml/min. The protein is detected by absorbance at 280 nm; fractions are collected at 1.0 min intervals and the radioactivity is measured in a gamma scintillation counter.

Patients:

Histological proof of Hodgkin's Disease, Kaposi's Sarcoma, or Head & Neck Caner. Patient must have measurable disease and failed prior potentially curative treatment regimens. Chemotherapy, surgery, radiation therapy or combinations. Potentially curative protocols will take precedent over this protocol.

Patient will be at least 10 years of age, with a life expectancy of over 3 months, and not pregnant. Patients will be advised to practice effective birth control during study.

Zubrod performance status 0, 1 or 2.

Patients must have adequate organ function as defined by: Granulocyte count $\geq$ 1500/mm^3; Platelet count $\geq$ 100,000/mm^3; Bilirubin $\leq$ 2.0, higher values acceptable if caused by HD or KS; Creatinine $\leq$ 2.0.Patients with abnormal serum chemistry or organ function can still be eligible for study, if this is due to their malignancy (primary or metastases).

Treatment:

Indium-111 labeled antiferritin IgM:

Under local anesthesia, three different needles (23 G) will be placed in three different parts of the tumor mass. Positioning of needle tip will be verified by two orthogonal X-rays films, real time ultrasound or CT. The selection of verification methods will depend on the anatomical location of the tumor. A volume of 0.5 cc containing 0.1 mg of antiferritin IgM labeled with 0.1 mCi of indium-111 will be utilized per injection site. Whole body scans will be obtained by gamma camera at 1, 20, 40, 120, and 165 hours after administration. A medium energy collimator will be utilized with collection centered at the peaks of indium-111 (173, 247 KeV). SPECT scans of tumor bearing area will be performed at 40 and 165 hours. Blood and urine samples will be obtained at 1, 20, 40, 120 and 165 hours and checked for radioactivity levels. The first and second 24 hours after administration, total urine will be collected for radioactivity testing. The percent injected dose in first and second twenty four hour urine, will be determined. The amount of radiation activity present in the tumor in one hour will be determined. Effective and biological half-life of the radioimmunoconjugate in the tumor will be determined, then elimination of blood radioactivity will be determined: monophasic, biphasic $T\alpha$, $T\beta$ and α/β. If over 90% of the deposited dose is retained in the tumor with a biological half-life of over 50 hours, patients will proceed to the intralesional administration of yttrium-90 labeled antiferritin.

Yttrium-90 labeled antiferritin IgM:

The volume of the tumor mass will be determined by summation of all transverse CT slices encompassing the tumor. The activity for administration will be 0.1 mCi per gram of the tumor (assuming the density of the tumor is 1). Administration will be performed as described under 5.1. The same studies will be performed after administration as given under 5.1., with the exception of using a low energy collimator and peaking of counts below 147 KeV. The results obtained will be compared to the results under 5.1 to verify as well as possible that the indium-111 labeled IgM has the same biodistribution and pharmacokinetics as the yttruim-90 labeled IgM.

The yttrium administration will be repeated twice, with one week intervals if the tumor remains detectable. For all histological types of tumor

an activity escalation will follow if the first five patients do not show acute side effects. New activity will be 0.2 mCI of yttrium-90 labeled antiferritin per gram of tumor per administration X 3. Only for Hodgkin's Disease and Head & Neck cancer patients will a third higher activity (0.4 mCi of yttrium-90 labeled antiferritin per gram of tumor X 3) be utilized if the second level (0.2 mCi X 3) does not produce acute side effects in five patients. The radiation dose per tumor is anticipated to be 30, 60 and 120 Gy for the 0.1, 0.2 and 0.4 mCi activity levels respectively.

Retreatment:

If a patient response is obtained or a complete response followed by recurrence, the patient can be retreated at the same or a higher activity level. If acute or late side effects have occurred at intensity level 3 or more after the first cycle, patients will not be retreated but go off study.

Evaluation during study:

Early side effects:

Allergic or anaphylactic reactions to low dose (2.5 mg) antiferritin radioimmunoconjugate have not been observed in the past. Vital signs of patients will be checked every 15 minutes for 60 minutes after injection of the radioimmunoconjugate. Anti-antibody formation has been observed in one patient only in prior studies of more than 80 patients with Hodgkin's Disease after I.V. antiferritin.[11] Anti-antibody (HAMA) is anticipated in patients with KS and Head & Neck Cancer. HAMA were noted in patients with glioblastoma multiforme and did not interfere with new cycles of treatment. HAMA will be determined two months after administration of antiferritin IgM and prior to a new cycle.

Late side effects:

Serum sickness or immune complex disease has not been observed in previously studied Hodgkin's Disease patients (n>80). The radiation dose received by normal tissues or Hodgkin's Disease tissues from indium labeled immunoconjugate is within the diagnostic X-ray/nuclear medicine range (less than 2 rad) and will not lead to biological effects. The yttrium labeled immunoconjugate is anticipated to cause tumoricidal effects and possibly

some acute or delayed side effects. Side effect evaluation is described under section 8.0 - Evaluation of Toxicity.

Tumor response:

Responses will be evaluated by repeated physical examinations, CT scans, gallium-67 scans and/or indium-111 antiferritin scans. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) will be used as endpoints and defined as complete disappearance of all prior known disease (CR), a fifty percent decrease or more in the product of orthogonal tumor diameters in old tumor sites with no appearance of new lesions (PR), less than fifty percent decrease or less than twenty-five percent increase in the product of orthogonal tumor diameters (SD), more than twenty-five percent increase in product of orthogonal tumor diameters (PD).

Evaluation of Toxicity:

All toxicity grades should be 1 or 0 prior to restarting a cycle. Grade 3 or 4 toxicity for any toxicity endpoints will prevent further treatment on this protocol for that particular patient and influence the treatment of other patients as indicated in Section 11.0. Toxicity grades will be assigned according to common toxicity grading criteria. After completion of treatment cycle, patients will be reevaluated in follow up once a month for two months. Thereafter follow up will be every two months X 2, followed by every three months X 2. Thereafter patients will be off study.

Criteria for Tumor response:

Acute and late side effects will be assessed. Tumor responses and duration will be determined by restaging methods, that will be selected for each patient and consist of physical exam and a selection of repeat diagnostic radiology studies (chest X-ray, CT scan, head and neck, chest, abdomen, pelvis, gallium scan, bone scan, indium-111 antiferritin scan). Only those diagnostic studies will be repeated that can provide unique information, instead of only duplicating information already known by other tests. In addition, the cheapest possible diagnostic test will be selected first.

Statistical Considerations:

Five patients will be tested at the first activity level. If therapy is well tolerated, escalation will be performed on the next five patients. If this escalation is tolerated, the remaining patients will be treated at the third radioactivity level. The third activity escalation will not be performed for the patients with Kaposi's Sarcoma.

Stopping rules are for toxicity. If toxicity exceeds grade 2 for two patients per group of five, that part of the study will be stopped.

Criteria for Removal From The Study:

Patients will be removed from study, in the case of progressive disease or toxicity of grade 3 or more. Patients removed from study will be followed for survival and late toxicity.

The teachings of the references cited herein are specifically incorporated herein. Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description and are intended to be encompassed by the following claims.

We claim:

1. A method for reducing the size of solid tissue comprising

administering an antibody composition intralesionally or intracompartmentally, wherein the antibody composition will remain localized in an amount effective to kill tissue at the site or within the compartment,

wherein the antibody composition comprises

antibodies selectively binding the tissue to be killed and which are in a form remaining localized in the tissue or region in which they are administered and

cytotoxic agents which are coupled to the antibodies.

2. The method of claim 1 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG, immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

3. The method of claim 1 wherein the cytotoxic agents are selected from the group of molecules consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

4. The method of claim 3 wherein the cytotoxic agents are cytotoxic radioisotopes.

5. The method of claim 3 wherein the cytotoxic agents are toxins.

6. The method of claim 2 wherein the antibodies are IgM.

7. The method of claim 1 wherein the antibodies comprise a conjugate which couples the cytotoxic agentss to the antibodies.

8. The method of claim 1 further comprising administering a diagnostic agent targeted to the same tissue as the cytotoxic agents, wherein the diagnostic agent is administered prior to administration of the cytotoxic agents, and detecting binding of the diagnostic agent to the tissue.

9. The method of claim 8 wherein the diagnostic agent is antibodies which selectively bind to the tissue to be killed labeled with a diagnostically detectable radioisotope.

10. The method of claim 1 wherein the tissue to be killed is solid tumor tissue.

11. The method of claim 10 wherein the tumors are selected from the group consisting of adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas.

12. The method of claim 1 wherein the tissue to be killed is endometriosis.

13. The method of claim 1 wherein the regions or compartments to be treated are selected from the group consisting of intrapleural compartments, head and neck cancer, breast, ovary, peritoneum, brain, and prostrate.

14. The method of claim 1 wherein the antibody composition is administered intraperitoneally.

15. The method of claim 1 wherein the antibody composition is administered intralesionally.

16. A method for treating disorders characterized by deposition of immune complex, inflammation and overproliferation of tissue comprising

administering at the site of immune complex deposition antibodies having cytotoxic agents coupled thereto.

17. The method of claim 16 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG, immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

18. The method of claim 16 wherein the cytotoxic agents are selected from the group of molecules consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

19. The method of claim 18 wherein the cytotoxic agents are cytotoxic radioisotopes and the disorder is rheumatoid arthritis.

20. An antibody composition comprising

antibodies selectively binding the tissue to be killed and which are in a form remaining localized in the tissue or region in which they are administered and

cytotoxic agents coupled to the antibodies.

21. The composition of claim 20 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG,

immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

22. The composition of claim 20 wherein the cytotoxic molecules are selected from the group consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

23. The composition of claim 22 wherein the cytotoxic agents are cytotoxic radioisotopes.

24. The composition of claim 22 wherein the cytotoxic agents are toxins.

25. The composition of claim 20 wherein the antibodies are IgM.

26. The composition of claim 20 wherein the antibodies comprise a conjugate which couples the cytotoxic agents to the antibodies.

27. The composition of claim 26 wherein the conjugate is diethylenetriaminepentaacetic acid compound labelled with a radioisotope.

28. The composition of claim 20 in a kit further comprising a diagnostic agent targeted to the same tissue as the cytotoxic agents.

29. The composition of claim 20 wherein the antibodies are targeted to a tumor selected from the group consisting of adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas.

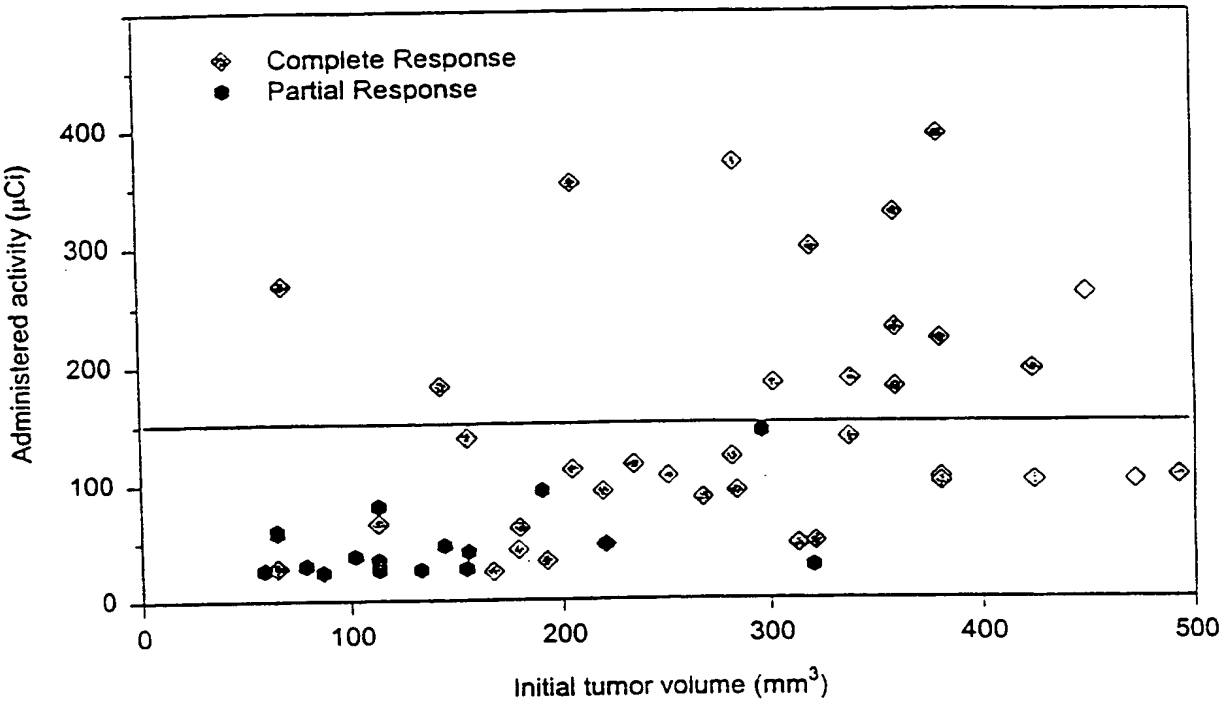


Figure 1 Tumor response following i.l. administration of ^{90}Y-labeled CR4E8. The difference in tumor response above and below 150 μCi is statistically significant, $P = 0.001$.

FIGURE 1

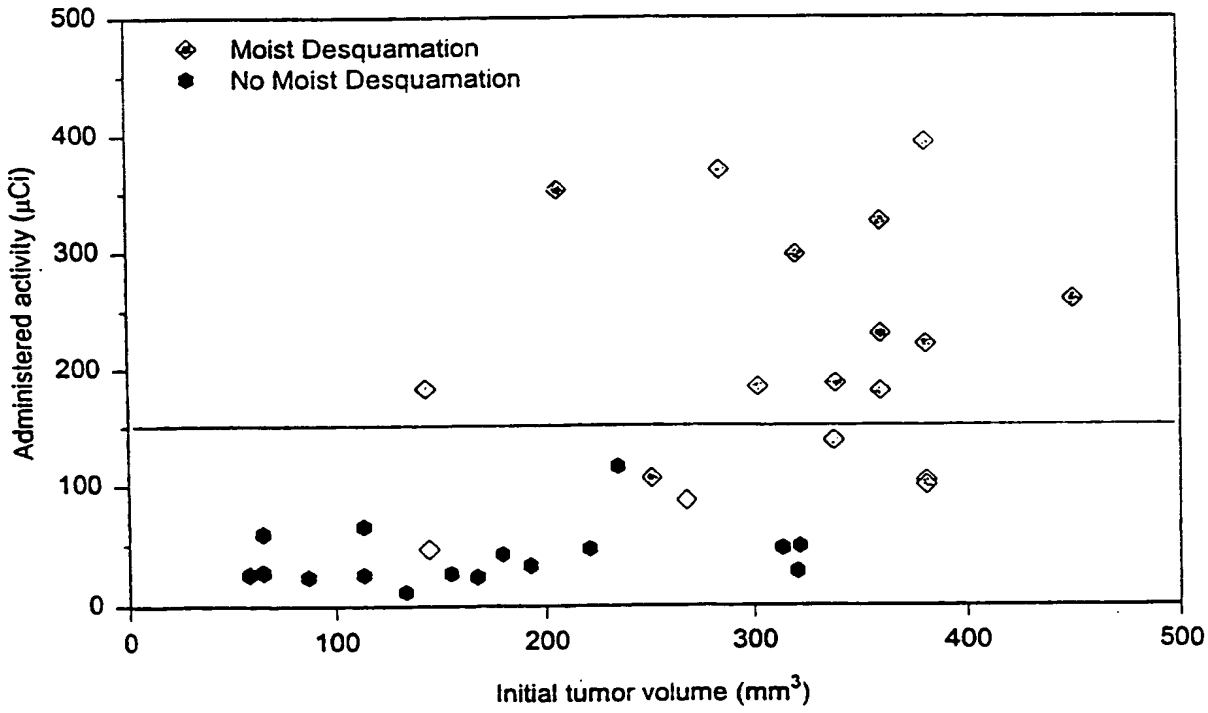


Figure 2. Occurenc of moist desquamation in mice treated with i.l. ^{90}Y-labeled CR4E8. The difference in occurence of moist desquamation above and below 150 μCi is statistically significant, $P = 0.00002$.

FIGURE 2

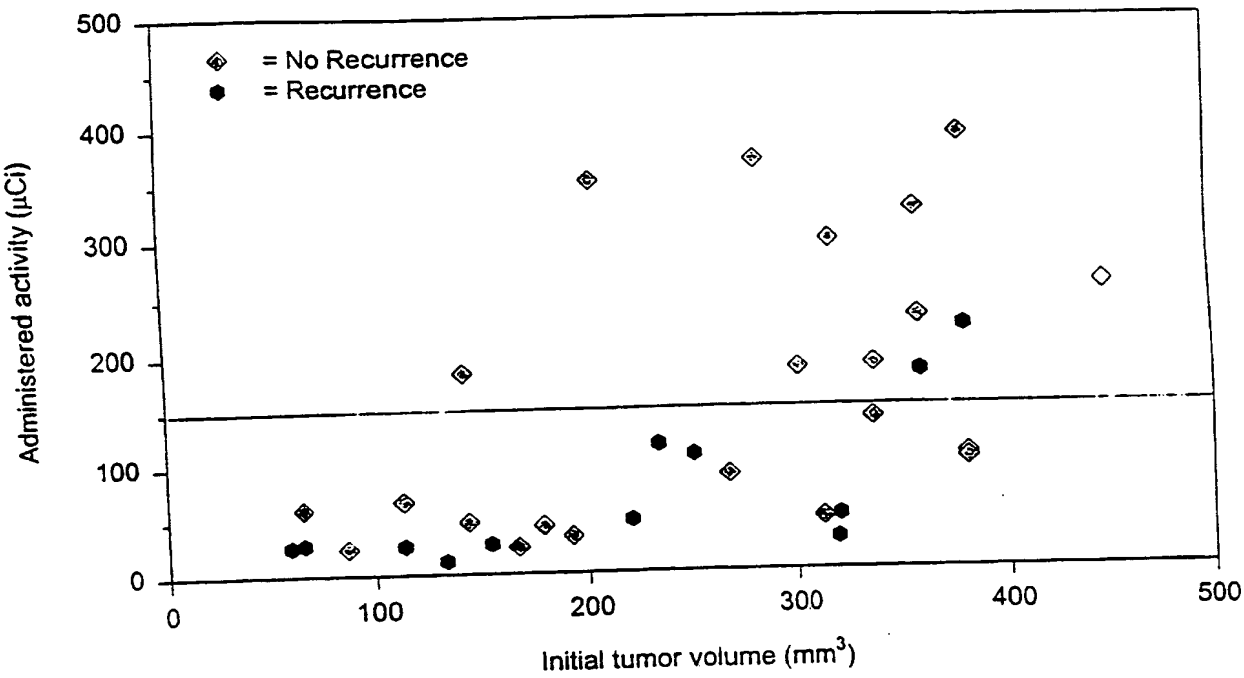


Figure 3. Incidence of tumor recurrence during duration of experiment (7.2 months) in mice treated with I.I. ^{90}Y-labeled CR4E8. The difference in rate of tumor recurrence above and below 150 µCi is statistically significant, P = 0. 039.

FIGURE 3

CORRECTED VERSION*

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(54) Title: USE OF RADIOLABELED MONOCLONAL IgM IN THERAPY FOR CANCER AND AUTOIMMUNE DISEASE

(57) Abstract

It has been discovered that large antibody aggregates or molecules, such as IgM or conjugated IgG or IgG fusion proteins, can be used to treat tumors by intracompartmental or intratumoral administration of anti-tumor antibody coupled to a toxin. The method can also be used in the treatment of certain disorders characterized by deposition of immune complex, for example, rheumatoid arthritis. In the preferred embodiment, the antibody is IgM and the toxin is a radioisotope, most preferably ^{111}In-labelled IgM or ^{90}Y-labelled IgM. Examples demonstrate effectiveness in mice models.

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USE OF RADIOLABELED MONOCLONAL IGM IN THERAPY FOR CANCER AND AUTOIMMUNE DISEASE

The present invention is generally in the area of cancer and autoimmune disease therapy using radiolabeled IgM antibodies specific for tumor or antibody producing cells.

The United States government has certain rights in this invention by virtue of National Institutes of Health grants CA 51161 and CA 16672.

Background of the Invention

Cancer is a major health problem. Two populations of human cancer patients are highlighted as examples.

Head and Neck Cancer

Each year in the United States there are approximately 53,000 new cases of cancer of the head and neck. Parker, et al., CA Cancer J. Clin. 65: 5-27 (1996). Over 90% are squamous cell carcinomas that originate on the mucosal surface of the upper aerodigestive tract. Shah, et al. CA Cancer J. Clin. 45: 352-368 (1995). At the time of presentation fewer than 10% of patients have distant metatases and only one-third of patients have small primary tumors less than four cm diameter that can be cured with local radiation therapy or surgery. Haraf, et al., Head and Neck Cancer: Basic and Clinical Aspects, pp. 173-198, Editors W.K. Hong and R.S. Weber, Kluwer, Norwell, MA (1994). Advanced disease, which is characterized by large primary tumors and regional lymph node involvement, often requires higher doses of radiation for cure than can be tolerated by the surrounding normal tissues and while bulky disease can be removed by radical surgery, the procedure is often mutilating and accompanied by problems associated with prolonged rehabilitation and residual morbidity.

Radiation therapy and surgery are frequently combined for patients with advanced disease, but 60% of them will still experience a local recurrence. Tupchong, et al. Int. J. Radiat. Oncol. Biol. Phys. 20: 21-28, (1991).

Ovarian Carcinoma

Ovarian carcinoma is the fourth most common cause of death from cancer in women, with 14,200 deaths annually in the U.S. (146Neijt, N. Engl. J. Med. 334, 50-51 (1996); Parker, et al., CA Cancer J. Clin. 199, 47:5-27 (1997)). The 5-year survival rate ranges from 50% for patients with tumors confined to the ovaries to less than 20% for patients with stage 3 and 4 disease (Teneriello, CA Cancer J. Clin. 45:71-87 (1995). At diagnosis, 75-85% of all patients have advanced disease (McGuire, Cancer (suppl) 71:1541-150 (1993)). Current first-line therapy for these patients consists of cytoreductive surgery followed by i.v. platinum-based chemotherapy that now includes paclitaxel. Response rates range from 60%-80% (McGuire, et al., N. Engl. J. Med. 334:1-6 (1996); Christian and Trible Gynecol. Oncol. 55:S143-S150 (1994); Thigpen, et al., Semin. Oncol. 18:248-154 (1991)); however, most of these patients will relapse.

The observation that most patients have disease in the peritoneal cavity and that it is the most common site of relapse has focused attention on regional treatment approaches. Intraperitoneal chemotherapy, i.p. colloidal ^{32}P, and external beam radiotherapy of the entire abdomen have been reported (Markman, Semin. Oncol. 18, 248-254 (1991); Ozols, et al., Epithelial ovarian cancer. In: Principles and Practice of Gynecologic Oncology. Hoskins, et al., (eds.) (J.B. Lippincott, Philadelphia, 1992) pp. 731-781). Tumor response and prolongation of survival are limited to patients with microscopic or very small macroscopic disease. Tissue penetration by i.p. administered chemotherapeutic agents is confined to the outer 1-2 mm of the tumor surface (Los, et al., Cancer Res. 49, 3380-3384 (1989); Ozols, et al., Cancer Res. 39, 3209-3214 (1979)). Most chemotherapy drugs have short half-lives in the peritoneal cavity, which is directly proportional to their small molecular size (Dedrick, et al., Cancer Treat. Rep. 62:1-11 (197)). Intraperitoneal colloidal ^{32}P nonselectively irradiates the surface of the peritoneal cavity (Boye, et al., Br. J. Radiol. 57:395-402 (1984); Currie, et al., Gynecol. Oncol. 12:193-218 (1981)). It is also prone to aggregation that produces inhomogenous dose distributions (Kaplan, et al., Radiology 138:683-688 (1981)). The presence of

radiosensitive organs in the abdominal region prevents the delivery of high doses of external beam radiation. The probability of eradicating measurable disease (cancer nodules greater than or equal to 5 mm) with low-dose external beam radiation is small.

Chemoresistant malignant cells usually respond to radiation and prior radiation therapy does not induce radioresistance. Intraperitoneal administration of murine IgG radiolabeled with ^{90}Y has demonstrated efficacy as an adjuvant (Stewart, et al., J. Clin. Oncol. 1941-1950 (1990); Maraveyas, et al., Cancer (suppl) 73:1067-1075 (1994)). However, myelotoxicity prevented dose escalation beyond 30 mCi of activity.

New applications of radioimmunotherapy can make an important contribution to the solution of the oncological problems highlighted in these examples.

Radioimmunotherapy (RIT)

RIT employs an antibody that recognizes a tumor-associated antigen to selectively deliver therapeutic radiation to a tumor. The specificity and high affinity that antibodies display towards their antigens led to optimistic expectations that radiolabeled antibodies would be "magic bullets" for cancer with very high therapeutic ratios. Pressman D., The Handbook of Cancer Immunology, Waters H (ed), Vol 5, 30-50 (Garland STPM Press, New York, 1978). This initial enthusiasm ignored the pharmacologic restraints imposed upon delivering such large molecules through the blood stream to tumors. Consequently, clinical RIT trials, particularly those for solid tumors, have been disappointing. Research has continued in order to understand the limitations of these reagents and to develop improved reagents and methodologies. RIT is now on the verge of making a large impact in the systemic treatment of hematologic malignancies, but it remains ineffective in the treatment of solid tumors.

Systemic or Intravenous RIT

Intravenous (i.v.) administration of radiolabeled murine IgG for treatment of solid tumors has been limited by low tumor deposition of radioactivity, myelotoxicity and induction of human anti-mouse antibodies (HAMA). Deposition of radioactivity in tumors following i.v. administration is frequently in the range of between 0.001 and 0.01% injected dose per gram (%ID/g), which rarely produces a tumor response. Epenetos, et al., Cancer Res. 46: 3183-3191(1988). Myelotoxicity is frequently the dose-limiting normal tissue toxicity after i.v. RIT through irradiation of bone marrow cells by blood borne immunoconjugate. Stein, et al., Br. J. Haematol. 80: 69-76, (1992); Vriesendorp, et al., Exp. Hematol. 24, 1183-1190 (1996). Most monoclonal antibodies used clinically are of murine origin and frequently induce the formation of human anti-mouse antibodies (HAMA) and an anti-antibody response in immunocompetent patients. Radioimmunoconjugates upon readministration can be bound by the anti-antibodies and prevented from reaching tumor. Additionally, these immune complexes are rapidly cleared by the reticuloendothelial system which may significantly increase the radiation dose to the liver and spleen. Immunosuppression and plasmapheresis of patients have been introduced to circumvent the induction of HAMA, but with limited success.

Systemic RIT utilizing radiolabeled IgG for hematologic malignancies has been more successful than RIT for solid tumors. However, the same limitations apply: low tumor targeting, myelotoxicity and to a lesser extent HAMA formation. Promising results have been obtained with systemic RIT for patients with Hodgkin's disease (Tupchong, et al. Int. J. Radiat. Oncol. Biol. Phys. 20, 21-28-1991; Vriesendorp, et al., Cancer Res. 55(suppl):5888s-5892s (1995)) with B-cell lymphomas (Vriesendorp, et al., Exp. Hematol. 24, 1183-1190 (1996), Wahl, et al., J. Nucl. Med. 28, 1736-1744 (1987), Waldmann, et al., Metabolism of immunoglobulins In: Progress in Allergy, 13, p. 35 Editors: Kallos, et al., (Karger, NY 1969), and Weinstein, et al., Science 222, 423-426 (1983)) and with T-cell lymphomas (Wheldon, Nucl. Med. Commun. 14, 408-410 (1993)).

Intracompartmental RIT

In view of the low level of tumor response following systemic RIT for patients with solid tumors, intracompartmental RIT has been attempted using radiolabeled IgG or its fragments. Small numbers of human cancer patients with a variety of solid tumors, such as in colon cancer, glioma, and ovarian cancer have been treated through the direct administration to the tumor or physical cavity that contains tumor. The total response rate for the combined quoted studies was 31%. Riva P. et al., Int J Biol Markers 8: 192-197 (1993). Papanastassiou V. et al., Br J Cancer 67: 144-151 (1993). Finkler N.J. et al., Gynecol Oncol 34: 339-344 (1989).

Even though intracompartmental administration of radiolabeled murine IgG can produce higher tumor deposition of radioactivity (Schlom, et al., Cancer Res. (Suppl) 50, 820s-827s (1990), Malamitsi, et al., J. Nuc. Med. 29, 1920-1925 (1988), and Haisma, et al., Am. J. Obstet. Gynecol. 159, 843-848 (1988)) and higher rates of tumor responses than i.v. administration, deposition of radioactivity in tumors remains low, myelotoxicity occurs (Hopkins, et al., Radiother. Oncol. 34, 121-131 (1995), Maraveyas, et al., Cancer (suppl) 73, 1067-1075 (1994), Hird, et al., Br. J. Cancer 68, 403-406 (1993), Breitz, et al., J. Nucl. Med. 36, 754-761 (1995)) and HAMA are induced (Riva, et al., Init. J. Biol. Markers 8, 192-197 (1993), Finkler, et al., Gynecol. Oncol. 34, 339-344 (1989), Jacobs, et al., Obstet. Gynecol. 82, 586-593 (1993), Stewart, et al., J. Clin. Oncol. 8, 1941-1950 (1990), Maraveyas, et al., Cancer (suppl) 73, 1067-1075 (1994), and Hird, et al., Br. J. Cancer 68, 403-406 (1993)).

It is therefore an object of the present invention to provide a means for more effective treatment of solid tumors.

It is a further object of the present invention to provide a means to lower immunogenicity compared to non-human antibodies and decrease myelotoxicity compared to treatment with anti-tumor IgG.

It is another object of the present invention to provide a means for treating solid tumors locally or intracompartmentally with high levels of deposition of radiation in the tumors.

Summary of the Invention

It has been discovered that large antibody aggregates or molecules, such as IgM or linked IgG (dimers, trimers, etc.) or IgG fusion proteins, can be used to treat tumors by intracompartmental or intratumoral administration of anti-tumor antibody coupled to a toxin. In the preferred embodiment, the antibody is IgM and the toxin is a radioisotope, most preferably ^{111}In-labelled IgM for diagnostic or dosimetric purposes or ^{90}Y-labelled IgM for therapeutic purposes.

Examples demonstrate effectiveness in mice models, and include protocols for treatment of peritoneal carcinomatosis and recurrent or persistent Hodgins' Disease, Kaposi Sarcoma, and head and neck cancer.

The antibody conjugates can also be used to treat autoimmune disease characterized by production of antibodies that produce immune complexes at defined surfaces, for example, Rheumatoid arthritis, where the conjugates diffuse into the joints to provide relief from the inflammation associated with the disorder.

Brief Description of the Drawings

Figure 1 is a graph of tumor response following i.l. administration of ^{90}Y-labeled CR4E8, as a function of tumor volume (mm^3) and the amount of radioactivity which is administered.

Figure 2 is a graph of the occurence of moist desquamation in mice treated with ^{90}Y-labeled CR4E8, as a function of initial tumor volume (mm^3) and the amount of radioactivity which is administered.

Figure 3 is a graph of the incidence of tumor recurrence in mice treated with i.l. ^{90}Y-labeled CR4E8, as a function of initial tumor volume (mm^3) and the amount of radioactivity which is administered.

Detailed Description of the Invention

The failure of i.v. RIT to effectively treat solid tumors stems primarily from the route of administration and the size and species of the immunoglobulin. Intravenous administration places a large amount of radioimmunoconjugate into the blood where it is diluted and distributed

throughout the circulatory system. Immunoglobulins are large molecules (150-950 kDa) that move primarily by convection (Jain, Scientific American 271, 58-65 (1994)). Tumors frequently have high interstitial pressure, which prevents the radioimmunoconjugates from entering the tumor interstitium by convection.

The limitations of systemic administration of IgG are avoided by (1) using intralesional or intracompartmental administration of antibody and (2) selection of an antibody that delivers a high dose of toxin, preferably radioisotope, and, due to the antibody's large size and affinity for tumor cells, stays in the general vicinity of where it is administered, as well as is targeted to the tumor.

IgG not conjugated to antigen, in circulation or accidentally deposited in perivascular spaces in patients with poor venous access, has a short half-life due to rapid catabolism and disappearance of small radioactivity moieties via circulation and urine elimination. IgG bound to antigen in blood or in tumor or IgM bound to tumor antigens is catabolized much slower. This underlines the potential of antigen specific IgM as a carrier for radiation. Unmodified IgM in circulation has a half-life of only five days, less than two half-lives of yttrium-90. The decrease in IgM catabolism by antigen binding allows for the delivery of more radiation and higher clinical efficacy. Simultaneously the tumor binding of IgM also keeps the radiation limited to the location where it is needed.

The best way to achieve high tumor deposition of radioactivity and avoid systemic toxicity, is to directly administer the radioimmunoconjugate at the disease site. This route of administration is typically applied to diseases that stay confined over their clinical history to a compartment. In this application, a compartment is defined as either a physical space like the peritoneal or pleural cavities or a large primary tumor. The intralesional (i.l.) or intraperitoneal (i.p.) administration of radiolabeled IgM provides much higher levels of radioactivity in tumors. Myelotoxicity is avoided since the intralesionally or intracompartmentally administered radioimmunoconjugate remains localized in the tumor or compartment. Initial clinical trials utilizing radiolabeled human IgM for diagnostic

purposes have demonstrated its low degree of immunogenicity. De Jager, R., et al., Sem. Nucl. Med. 23: 165-179, (1993). Other applications include diseases where a large tumor could be treated palliatively.

The reactivity of the antibody with tumor cell antigens coupled with the high avidity (10 antigen binding sites) and large mass of IgM (900 kDa) keeps the radioimmunoconjugate within the tumor. Because IgM is soluble, the radioimmunoconjugate should diffuse through the tumor over time. Thus, more selective high-dose radiation of irregular tumor volumes is possible with i.1. RIT than with conventional radiation therapy.

RIT can result in a mixture of immunological and radiation effects. Studies in patients with Hodgkin's disease clearly established the radiation aspects of RIT. Tumor masses that are not targeted do not shrink. Dose escalation increases the chance for a tumor response. Small fraction sizes are not effective. Tumor recurrences after RIT are not radioresistant. All these aspects are similar to observations made over the years in patients undergoing external beam radiation. This indicates that RIT has an effect based on and eqiuvalent to external beam radiation but in another form. Any pathological process in the body which is susceptible to radiation but cannot be treated successfully with it due to toxicity concerns, can therefore be treated with RIT, which by definition has a higher therapeutic ratio than other non specific delivery systems of radiation. Examples of other disorders include rheumatoid arthritis and restenois, as discussed in more detail below.

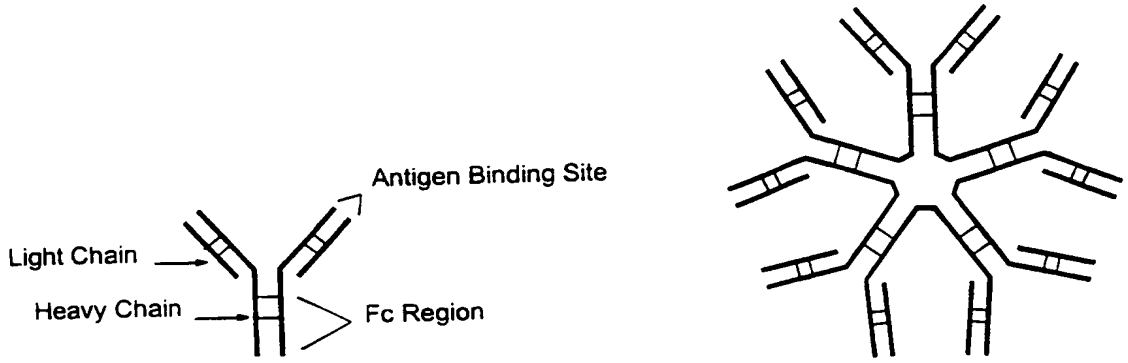
Components of RIT

In vitro

Several components determine the design and synthesis of a therapeutic radioimmunoconjugate: tumor antigen, an antibody that is immunoreactive with the tumor antigen, a cytotoxic agent such as a radioisotope and means for coupling the cytotoxic agent to the antibody which does not alter immunoreactivity of the antibody. These *in vitro* components are discussed below, followed by a discussion of the variables that influence the *in vivo* distribution of the radioimmunoconjugate after administration.

Antibodies

There are five classes of antibodies: IgA, IgD, IgE, IgG, and IgM. Of these, only IgG and IgM have been used as radioimmunoconjugates. This is, in part, due to technical reasons since splenic B-cells from mice generate hybridomas that usually secrete IgG_1 and IgG_{2a} (Keenan, et al., J. Nucl. Med. 26:531-537 (1985)) while hybridomas produced from human lymphocytes frequently secrete IgM. IgG and IgM are very similar in structure and function. IgG is a monomer, while IgM is a pentamer composed of five identical monomers. Each monomer is composed of two identical heavy chains and two identical light chains. The light chains are composed of two domains: the variable domain on the amino terminus, which contains the antigen-binding site, and a constant domain on the carboxy terminus. The heavy chain has a similar arrangement with one variable domain on the amino terminus end followed by three constant domains for IgG and four constant domains for IgM. Each heavy chain is bound to a light chain through disulfide bonds. This association produces a more effective antigen-binding site that gives the antibody its specificity. The last two constant domains of the heavy chain contain the Fc region, which regulates effector functions of the antibody. Two strands composed of a light chain and a heavy chain are linked together through the heavy chains by disulfide bonds to form the monomer. IgM possesses an additional polypeptide chain, the J chain, that is thought to assist the polymerization of the pentamer.



IgG

~150 kDa

IgM

~950 kDa

Although described primarily with reference to IgM, it is to be understood that other antibody molecules can be substituted for IgM,

including conjugates or polymers of IgG, immunoglobulin fusion proteins, and recombinant fragments or humanized IgM antibodies, can be delivered to a site or compartment where the antibody molecules will remain localized. These are collectively referred to herein as "IgM" unless specifically noted.

Polyclonal Antibodies

IgG and IgM can be isolated from the sera of immunized animals. These antibodies are termed polyclonal in that they arise from many different clones of antigen-stimulated B-cells. The percentage of the polyclonal antibodies that is reactive with the antigen used for immunization is low, (less than 20%). Affinity purification is sometimes used to increase this percentage. Polyclonal antibodies are now used infrequently since it takes several months of immunizations to generate antibodies of sufficient specificity and titer, and there is lot-to-lot variability.

Monoclonal Antibodies

The introduction of hybridoma technology allowed the large-scale production, through cell culture techniques, of monoclonal antibodies with defined specificity and very high reactivity. Most monoclonal antibodies developed for clinical use are murine in origin owing to the initial requirement of splenic tissue as a source of stimulated B-cells. Mouse monoclonal antibodies frequently induce HAMA in immunocompetent patients. Many attempts have been made at reducing the adverse effects of HAMA. Some are pharmacological like plasmapheresis (Zimmer, et al., J. Nucl. Med. 29:174-180 (1988)) and immunosuppresion of the patient (Riva, et al., Int. J. Biol. Markers 8:192-197 (1993); Weiden, et al., Cancer 73:1093-1097 (1994)). Others use enzymes like pepsin and papain to cleave the Fc region from IgG to produce smaller fragments for administration that retain the ability to bind antigen but lack the effector portion of the immunoglobulin (Carrasquillo, et al., Cancer Treat. Rep. 68:317-328 (1984)). More recently, genetic engineering has been employed to replace immunogenic regions of a murine antibody with human regions. In the first attempts, the constant domains of a mouse antibody were replaced with the constant domains of a human antibody (Boulianne, et al., Nature 312:643-646 (1984); Morrison, et al. Proc. Natl. Acad. Sci. 81:6851-6855 (1984)).

The resulting antibodies are termed chimeric antibodies and can still be immunogenic (Khazaeli, et al., Cancer Res. 51:5461-5466 (1991)). Further refinements through the grafting of the hypervariable regions of a mouse antibody onto the variable domains of a human antibody have produced what are termed humanized antibodies that may possess reduced immunogenicity (Jones, et al., Nature 321:522-525 (1986)).

Human antibodies are the preferred choice as the least immunogenic antibody for human patients (De Jager, et al. Semin. Nucl. Med. 23:165-179 (1993)). Additionally, human antibodies may be less reactive with normal human tissue antigens and be more specific for human tumor antigens. The realization that patients with malignancies may have stimulated B-cells in their tumor-draining lymph nodes and in their peripheral blood that produce immunoglobulins against their cancer has provided an important source of human B-cells (Freedman, et al., Hybridoman 10:21-33 (1991); Chen, et al., Hum. Antibodies Hybridomas 5:131-142 (1994); Nishinaka, et al., Cancer Res.56:5666-5671 (1996)). After fusion with a myeloma cell, many human hybridomas are unstable and produce only low titers of immunoglobulins. Production problems may be overcome by genetic engineering techniques such as the construction of yeast artificial chromosomes and their expression in cell culture or transgenic animals (Pennisi, Sci. News 143:360-363 (1993)).

Antigens

The tumor antigens used to target radioimmunoconjugates are almost exclusively tumor-associated antigens. These are not specific markers for tumor cells in most cases; rather, they are overexpressed on tumor cells compared with normal tissue, or they are found in association with normal fetal tissue, such as CEA (Gold, et al., J. Exp. Med. 122, 467-481 (1965)), AFP (Abelev, Adv. Cancer Res. 14, 295-350 (1971)) or with normal progenitor cells of that organ in the adult (CEA). Tumor antigens can be localized in the tumor interstitium, on the tumor cell membrane, or in the tumor cell cytoplasm or nucleus.

The location of the tumor antigen directs the selection of the route of administration of the radioimmunoconjugate and the type of therapeutic

radioisotope needed for effective tumor sterilization. Antigens that are found on leukemia cells in circulation and antigens expressed on tumor neovasculature are readily accessible to i.v. administered reagents. Antigens that are expressed on the surface of tumor cells are readily accessible to i.l. or i.p. administered radioimmunoconjugates. Antigens secreted into the tumor interstitium are most accessible to i.l. administration.

The preferred anti-tumor antibodies are human IgM that recognize tumor associated antigens that are either expressed on the tumor cell membrane or secreted into the tumor interstitium. Table 1 lists the preferred antibodies, disease applications, and sources.

TABLE 1: ANTIBODIES FOR USE IN IgM RIT.

Designation	Origin	Antigen size/location	Disease application
AC6C3-2B12[1]	human	32 kDa cell membrane (SKOV3, human ovarian cell line)	Adenoma (breast, colon, ovarian)
CR4E8[2]	human	55 kDa cell membrane (SW756, human cervical carcinoma cell line)	Squamous cell (cervical, head carcinoma and neck) prostate
ACC 0101	murine	ferritin (440 kDa), interstitium	Hodgkin's disease, head and neck, hepatoma, sarcoma
Kaposi's			
L72[3]	human	GD_2 (ganglioside) cell membrane	glioma, melanoma, neuroblastoma
126-4[4]	murine	GD_2 (ganglioside) cell membrane	glioma, melanoma, neuroblastoma

References:

1 Freedman RS, Ioannides CG, Tomasovic B, et al. Development of a cell surface reacting human monoclonal antibody recognizing ovarian and certain other malignancies. Hybridoma, 1991, 10: 21-33.

2 Chen P-F, Freedman RS, Chernajovsky Y, et al. Amplification of immunoglobulin transcripts by the non-palindromic adapter polymerase chain reaction (NPA_PCR). Nucleotide sequence analysis of two human monoclonal antibodies recognizing two cell surface antigens expressed in ovarian, cervix, breast, colon and other carcinomas. Hum Antibod Hybridomas, 1994; 5: 131-142.

3 Katano M, Jien M, Irie Reiko. Human monoclonal antibody to ganglioside GD2-inhibited human melanoma xenograft. 1984; Eur J Cancer Clin Oncol, 20; 1053-1059.

4 Schulz G, Cheresh DA, Varki NM, et al. Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. Cancer Res, 1984, 44: 5914-5920.

Cytotoxic Compounds: Radioisotopes

Although described primarily with reference to radioisotopes, especially indium ("In"), which is useful for diagnostic purposes, and yttrium ("Y"), which is cytotoxic, other substances which kill cells can be substituted for the radioisotope. The radioisotopes are preferred since they are small and well characterized, and can be used as diagnostics and followed after administration using standard non-invasive radioimaging techniques. Non-radioisotopes can include toxins and substances which elicit the host to attack the tumor cells, as well as synthetic or natural chemotherapeutic drugs (Halpern, et al., J. Nucl. Med. 29:1688-1696 (1988); Quadri, et al., Nucl. Med. Biol. 20:559-570 (1993); Wang, et al., Radiat. Res. 141:292-302 (1995)), oligonucleotides (Mujoo, et al., Oncogene 12:1617-1623 (1996)), cytokines (Markman, Semin. Oncol. 18:248-254 (1991); Dedrick, et al., Cancer. Treat Rep. 62:1-11 (1978)), and radioactive colloids (Rowlinson, et al., Cancer Res. 47:6528-6531 (1987)). These can be conjugated to the antibody using standard chemical techniques, or in some cases, using recombinant technology, for example, fusion proteins.

As radioisotopes decay, they emit characteristic photons or particles or both. Photons, commonly referred to as gamma rays, are penetrating. If their energy level is high enough, they can travel through the body and be detected by diagnostic instrumentation. Radioisotopes that emit photons can be attached to an antibody and used for diagnostic imaging. This application is termed radioimmunoscintigraphy (RIS).

The shorter the distance between the antigen and the target (tumor cell DNA), the shorter the required range of emission of the radioisotope. Auger electrons have a very short path length (5-10 nm) and need to be internalized to be cytotoxic (Adelstein, et al., Nucl. Med. Biol. 14:165-169 (1987)). Only antibodies that are internalized after binding to a cell should be considered for radioisotopes that emit Auger electrons. Alpha particles need to be close to a cell (within 3-4 cell diameters) to be effective (Vriesendorp, et al., Radioimmunoglobulin therapy. In: High Dose Cancer Therapy. Armitage, et al. (eds). (Williams & Wilkins, Baltimore, MD 1992) pp. 84-123), so antibodies that target interstitial antigens would not be

desirable. Both Auger electrons and alpha emitters have high selectivity because their short-range emission will not irradiate neighboring normal cells. Conversely, untargeted neighboring malignant cells will also avoid irradiation. Tumor sterilization by radioimmunoconjugates carrying Auger electron or alpha emitters can only be achieved by the targeting of all clonogenic tumor cells.

Long-range beta emissions are advantageous as they will produce more homogenous distribution of radiation within a tumor, preventing "cold" spots by lethally irradiating untargeted cells through cross-fire of emissions from neighboring areas (Wheldon, Nucl. med. Commun. 14:408-410 (1993)). The drawback of long-range emissions is that they are less selective and less effective for treating tumor masses that are smaller than the path length of the beta particles. With smaller masses, most of the energy is deposited outside of the tumor mass. This increases normal tissue toxicity and reduces the tumor dose.

The radiometals ^{111}In and ^{90}Y are, respectively, pure γ- and pure β-emitters. Iodine-125, the most commonly used emitter of Auger electrons, has a half-life of 60 days and frequently is released by the immunoconjugate *in vivo* (dehalogenation) (Vriesendorp, et al., 1992). The most commonly considered alpha emitters for clinical use, astatine-211 and bismuth-212, have short half-lives (7.2 h and 1.0 h, respectively) and decay into radioactive isotopes, that may not be retained by the immunoconjugate after the first alpha emission (Wilbur, Antibiot. Immunoconjug. Radiopharm. 4:85-97 (1991)). The use of an immunoconjugate radiolabeled with ^{111}In has been proposed to predict the behavior of the poorly imageable ^{90}Y-labeled immunoconjugate (Korngold, et al., Cancer Res. 20:1488-1494 (1960); Welt, et al., J. Clin. Oncol. 12:1561-1571 (1994); Breitz, et al., J. Nucl. Med. 33:1099-1112 (1992); Vriesendorp, et al., Cancer Res. (suppl) 55:5888s-5892s (1995)). Previous studies using stable radiometal chelation have demonstrated similar biodistributions for radioimmunoconjugates labeled with ^{111}In and ^{90}Y (Welt, et al., J. Clin. Oncol. 12:1561-1571 (1994); Breitz, et al., J. Nucl. Med. 33:1099-1112 (1992)). In practice, immunoscintigraphy of patients after administration of the ^{111}In-labeled antibody would allow the

physician to screen patients for tumor localization and determine the *in vivo* stability of the radioimmunoconjugate. Then, only patients expected to benefit from treatment would receive the antibody again, which is now labeled with a therapeutic quantity of ^{90}Y. This treatment approach would allow patients to be treated as outpatients because the administered activity of ^{111}In is low and the emissions from ^{90}Y are largely absorbed in the vicinity of the tumor.

Beta-emitting isotopes for RIT can be either mixed beta and gamma emitters like ^{131}I, with 90% gamma, and ^{67}Cu, with 60% gamma (Schubiger, et al., Bioconjug. Chem. 7:165-179 (1996)) or pure beta-emitters like ^{90}Y and ^{32}P. Both types of radioisotopes have their supporters. Advocates of radioisotopes with mixed emissions like the simplicity of using the same radioimmunoconjugate at a low activity to demonstrate tumor targeting with gamma camera imaging and, by escalating the activity, administer therapy in the next treatment. This way, the pharmacokinetics of the radio-immunoconjugate should be identical for both administrations, and the first administration should be predictive for the second administration if the protein dose is held constant. However, this simplicity comes at the cost of exposing patients to high doses of gamma rays when therapeutic activities are employed. In addition, elaborate inpatient management becomes necessary to prevent the exposure of medical personal and the general public to unacceptable levels of radiation.

Attachment of Cytotoxic compound or Radioisotope to Antibody

Some radioisotopes can be attached directly to the antibody; others require an indirect form of attachment. The radioisotopes ^{125}I, ^{131}I, ^{99m}Tc, ^{186}Re and ^{188}Re can be covalently bound to proteins (including antibodies) through amino acid functional groups. For radioactive iodine it is usually through the phenolic group found on tyrosine. There are numerous methods to accomplish this: chloramine-T (Greenwood, et al. Biochem J. 89: 114-123 (1963)); and Iodogen (Salacinski, et al. Anal. Biochem. 117: 136-146 (1981)). Tc and Re can be covalently bound through the sulfhydryl group of cysteine (Griffiths, et al. Cancer Res. 51: 4594-4602 (1991)). The problem with most of the techniques is that the body has efficient methods to break

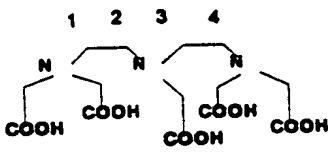
these covalent bonds, releasing the radioisotopes back into the circulatory system. Generally, these methods are acceptable for imaging purposes (^{99m}Tc), but not for therapeutic purposes.

Numerous types of cytotoxic compounds can be joined to proteins through the use of a reactive group on the cytotoxic compound or through the use of a cross-linking agent. A common reactive group that will form a stable covalent bond in vivo with an amine is isothiocyanate (Means, et al. Chemical modifications of proteins (Holden-Day, San Francisco 1971) pp. 105-110). This group preferentially reacts with the ε-amine group of lysine. Maleimide is a commonly used reactive group to form a stable *in vivo* covalent bond with the sulfhydryl group on cysteine (Ji. Methods Enzymol 91: 580-609 (1983)). Monoclonal antibodies are incapable of forming covalent bonds with radiometal ions, but they can be attached to the antibody indirectly through the use of chelating agents that are covalently linked to the antibodies. Chelating agents can be attached through amines (Meares, et al., Anal. Biochem. 142:68-78 (1984)) and sulfhydral groups (Koyama Chem. Abstr. 120:217262t (1994)) of amino acid residues and also through carbohydrate groups (Rodwell, et al., Proc. Natl. Acad. Sci. 83:2632-2636 (1986); Quadri, et al., Nucl. Med. Biol. 20:559-570 (1993)). Since these chelating agents contain two types of functional groups, one to bind metal ions and the other to joining the chelate to the antibody, they are commonly referred as bifunctional chelating agents (Sundberg, et al., Nature 250:587-588 (1974)).

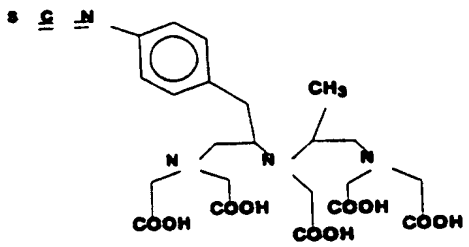
Crosslinking agents have two reactive functional groups and are classified as being homo or heterobifunctional. Examples of homobifunctional crosslinking agents include bismaleimidohexane (BMH) which is reactive with sulfhydryl groups (Chen, et al. J Biol Chem 266: 18237-18243 (1991) and ethylene glycolbis[succinimidylsucciate] EGS which is reactive with amino groups (Browning, et al., J. Immunol. 143: 1859-1867 (1989)). An example of a heterobifunctional crosslinker is m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Myers, et al. J. Immunol. Meth.121: 129-142 (1989)). These methodologies are simple and are commonly employed.

^{111}In and ^{90}Y are available as trivalent ions in aqueous hydrochloric acid (pH 1). They precipitate as insoluble hydroxides at pH greater than 3.4 for In and greater than 7 for Y (Brunner, et al., Radiometals and their chelates. In: Principles of Nuclear Medicine. Wagner, et al., (eds.) (2nd ed., Saunders, Philadelphia 1995)). Trivalent indium can be stabilized at neutral pH if a chelate with at least 6 donor ligands is used to saturate the coordination sites of the ion. The ionic radius of trivalent yttrium is 15% larger than that of indium and requires a chelating group that can donate at least 8 ligands. The polyamino-polycarboxylic ligand, diethylenetriaminepentaacetic acid (DTPA) has 8 ligands and will strongly chelate both indium and yttrium. The structure of DTPA is shown below. Initially, DTPA, as a cyclic anhydride, was bound to antibody by an amide bond formed between one of DTPA's carboxylic acids and an amine on the antibody (Krejcarek, et al., Biochem. Biophys. Res. Commun. 77:581-585 (1976); Hnatowich, et al., Science 220:613-615 (1983)). This chelating structure was unstable *in vivo* with yttrium (Hnatowich, et al., J. Nucl. Med. 29:1428-1434 (1988)) because the loss of the carboxyl group decreased the number of donor ligands to 7. Substitutions to the carbon backbone of the molecule were developed as a means to attach the chelating group to the antibody without losing one of the ligands necessary for binding (Brechbiel, et al., Inorg. Chem. 25:2772-2781 (1986); Brechibiel, et al., Bioconjugate Chem. 2:187-194 (1991); Gansow, Nucl. Med. Biol. 18:369-381 (1991); Quadri, et al., Bioorg. Med. Chem. Lett. 2:1661-1664 (1992)). Bifunctional chelating agents attached to an antibody in this manner demonstrate stable chelation of yttrium *in vitro* and *in vivo* (Kozak, et al., Cancer Res. 49:2639-2644 (1989)). The backbone-substituted DTPA described herein, 2B3M-DTPA (Quadri, et al., 1992)), is shown below. The numbers indicate the position of the benzyl group (2B) and the methyl group (3M). The methyl group on carbon 3 provides additional stability to the coordination complex (Margerum, et al., Kinetics and mechanisms of complex formation and ligand exchange. In: Coordination Chemistry, monograph 174, Martell (ed) vol. 2 (American Chemical Society, Washington, D.C. 1978) pp. 1-220). The benzyl group attached to carbon 2 acts as a linker to join the chelating

group to the antibody through a thiourea bond created from the reaction of the isothiocyanato group (S=C=N) with an amino group from the antibody (Meares, et al., Anal. Biochem. 142:68-78 (1984)). The linker is stable *in vivo* and, depending on the application of the radioimmunoconjugate, may increase normal tissue toxicity. Linker-chelate structures that are more labile in normal tissue than tumor have been developed to decrease normal tissue toxicity (Deshpande, et al., Nucl. Med. Biol. 16:587-597 (1989); Quadri, et al., J. Nucl. Med. 34:938-945 (1993)).



DTPA



2B3M-DTPA

1

Chelating structures for radiometals.

The location of chelating groups on the immunoglobulin depends on the location and accessibility of the functional groups with which the reactive group on the linker can form a covalent bond. The isothiocyanato group reacts preferentially with the epsilon amino groups of lysine. The number of chelating groups attached to an antibody is controlled by the stoichiometric ratio between the chelating group and the antibody when they are reacted together. Low numbers of chelating groups are desirable, typically one group for IgG and four groups for IgM, in order to lessen the chance of blocking the antigen-binding sites and keep the specific activity, once the immunoconjugate is radiolabeled, within a reasonable range (10 mCi/mg for ^{90}Y). Higher activities increase radiolysis, i.e. breakage of the bond between the chelate and the protein by beta emissions.

Other bifunctional chelating structures have been developed including hinge-specific (Ali, et al., Bioconjug. Chem. 7:576-583 (1996)) and macrocyclic structures (Li, et al., Bioconjug. Chem. 4:275-283 (1993); Cox, et al., J. Chem. Soc. Perkin Trans. 1:2567-2576 (1990); Moi, et al., J. Am. Chem. Soc. 110:6266 (1988)). Hinge-specific bifunctional chelators are attached to antibodies through the sulfhydryl groups and form a single covalent bridge between two heavy chains in the hinge region. This produces immunoconjugates with exactly one chelate in a known location away from the antigen-binding site. Additionally, these chelating groups can stabilize immunoconjugate fragments by replacing the labile disulfide bonds holding the fragments together. Unmodified $F(ab')_2$ fragments are quickly reduced *in vivo* to Fab' fragments that are rapidly deposited in kidney (Quadri, et al., J. Nucl. Med. 34:2152-2159 (1993)). The macrocyclic chelates are cyclic polyamino-polycarboxylates as opposed to an open chain like DTPA. The chelation complexes formed with the macrocyclics have higher thermodynamic stability than those formed with DTPA (Harrison, et al., Nucl. Med. Biol. 18:469-476 (1991)), but they suffer from low specific activities, awkward labeling procedures and immunogenicity (Kosmas, et al., Cancer Res. 52:904-911 (1992)).

Methods and Dosages Required for *In vivo* Treatment

The targeted cytotoxic molecules can be administered intralesionally or intraregionally, so that the molecules remain in the targeted area for a time sufficient for binding to the tissue to be killed. Although described primarily with reference to tumors, there are many other types of tissue which it may be desirable to kill, such as the overproliferative tissue characteristic of endometriosis, or joint linings in the case of RA or other disorders characterized by immune complex deposition, inflammation, and overproliferation of tissue. As discussed above, the targeting molecule is designed to have two characteristics: selective binding to the targeted tissue, and large size, low diffusibility out of the region or tissue into which it is administered. Intralesional administration will typically be into the tissue to be killed, usually a solid tumor. Intraregional administration will be into a cavity such as the peritoneum or lungs. Types of cancer which can be treated include adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas (recurrent or persistent Hodgins' Disease; other than Hodgkin's lymphoma). Regions or compartments to be treated include intrapleural compartments, head and neck cancer, breast, ovary, peritoneum (peritoneal carcinomatosis), brain, prostrate, as well as other solid tumors and overproliferative tissue such as endometriosis in the uterus and peritoneum.

After selecting an appropriate targeted cytotoxic molecule, preferably an antigen-antibody-radioisotope combination, for the disease to be treated, administration variables must be considered, such as route of administration, administered activity, single or fractionated administration, protein dose, specific activity, predosing with unlabeled antibody. In a preferred embodiment, the administered activity level can be based on those used in previous studies or dosimetric calculations performed using the data acquired with the diagnostic radioisotope in the RIS phase of RIT. A radioimmunoconjugate can be administered once or divided into smaller activities over a short (1-2 week) period of time (i.e., fractionated). Fractionation can increase the therapeutic index by decreasing normal tissue toxicity (Schlom, et al., J. Natl. Cancer Inst. 82:763-771 (1990); Meredith, et

al., J. Nucl. Med. 33:1648-1653 (1992)). Fractionation does not appear to decrease the effects of RIT on tumor, unless a long fractionation schedule and a rapidly proliferating tumor are studied (Molthoff, et al., Int. J. Cancer 50:474-480 (1992)). The radiobiological principles of cell kill differ slightly for low, decreasing dose rate radiation via RIT and high dose rate external beam radiation. However, the surviving fractions of clonogenic malignant cells (and supposedly also clonogenic normal tissue cells) are similar after similar doses of radiation from RIT or external beam (Vriesendorp, et al., Radioimmunoglobulin therapy. In: High Dose Cancer Therapy. Armitage, et al. (eds) (Williams and Wilkins, Baltimore 1992) pp. 84-123).

High protein doses can activate complement, which will in turn produce side effects such as fevers, chills and shortness of breath. With high protein levels, tumors cells may be killed through immunologic mechanisms like complement-dependent tumor cytolysis or antibody-mediated cellular cytolysis. The analysis of RIT effects on tumor becomes more complicated if the tumor response is due to a combination of immunologic and radiation effects. Higher levels of protein are also more immunogenic. In general, it is advantageous for initial clinical studies to use a low protein dose (approximately 5 mg per administration).

The specific activity of a radioimmunoconjugate relates to its immunoreactivity, stability and therapeutic efficacy. If the specific activity is too high, the large number of chelates required per immunoglobulin molecule might reduce immunoreactivity. A high specific activity increases radiolysis. On the other hand, too low of a specific activity may result in undertreatment of a tumor since unlabeled immunoconjugates will compete with radiolabeled immunoconjugates for antigen binding. This is particularly important with an antigen that is expressed at a low density on a tumor cell.

Predosing with cold antibody has increased tumor deposition of radioactivity in some clinical trials using i.v. administration (DeNardo, et al. Cancer 73:1023-1032 (1994)). This may occur through the saturation of receptor sites that are involved in the metabolism of immunoglobulins or

through immunologic mechanisms by which the tumor becomes more accessible to the radioimmunoconjugate.

Two Stage Imaging/ Therapy

The demonstration of biodistribution and tumor targeting of a radioimmunoconjugate through gamma camera imaging is a major strength of RIT that is not available for other cancer treatment modalities. Imaging can be used to screen patients so that only those patients expected to benefit from therapy receive the immunoconjugate labeled with a high activity of the therapeutic radioisotope. However, the diagnostic part of RIT can be separated from the therapeutic part of RIT by employing the same immunoconjugate twice but radiolabeled with a different radioisotope for each administration. For the diagnostic administration, the immunoconjugate would be radiolabeled with a pure gamma-emitting radioisotope like indium-111 (^{111}In) or technetium-99m (^{99m}Tc). Both of these isotopes emit gamma rays within the appropriate energy range for imaging, (100-250 keV). Energies below this range are not penetrating enough to reach an external imaging device. Higher energy levels are difficult to collimate and provide diagnostic images with poor resolution. The short-half life of ^{99m}Tc restricts its use to immunoconjugates with rapid tumor uptake. The use of ^{111}In-labeled immunoconjugate has been proposed to predict the *in vivo* behavior of an immunoconjugate radiolabeled with ^{90}Y, a pure beta-emitter, since they have similar half-lives and comparable chelation chemistry (Vriesendorp, et al., Cancer. Res. (suppl) 55:5888s-5892s (1995); Vriesendorp, et al., Radioimmunoglobulin therapy. 1992); DeNardo, et al., J. Nucl. Med. 36:829-836 (1995); Leichner, et al., Int. J. Radiat. Oncol. Biol. Phys. 14:1033-1042 (1988)).

An additional advantage of using two separate radioisotopes, one for imaging and one for therapy, is that it allows for outpatient treatment. The low amount of radioactivity used diagnostically does not represent a radiation hazard, while the radiation emitted by a therapeutic pure beta-emitter will largely be absorbed in the vicinity of the tumor. This treatment scheme is dependent on similar pharmacokinetics for both radiolabeled

reagents and requires a stable means of attaching both radiometals to the antibody, which is discussed below.

Implicit in a treatment program that uses tumor targeting as a selection criterion for treatment, is the presence of measurable disease, i.e. tumor with a diameter greater than 1 cm. Tumors smaller than 1 cm are usually not measurable by CT, by MRI or with an ^{111}In-labeled radioimmunoconjugate. For measurable disease (greater than 1 cm), ^{90}Y, with its long-range beta emission, has the advantage of providing a higher and more homogenous tumor dose than beta-emitters with a shorter range (Leichner, et al., Med. Phys. 20:529-534 (1993)).

Detailed protocols are described below for intraperitoneal administration of IgM for the treatment of periotoneal carcinomatosis and intralesional administration of anti-ferritin IgM for treatment of Hodgkins' disease, Kaposi's sarcoma, and head and neck cancer.

As described in the examples, the protocols are written as a two stage approach with ^{111}In-labeled IgM used to demonstrate tumor targeting and to provide dosimetric information and, when warranted, followed by therapy with the ^{90}Y-labeled IgM. For some applications like Kaposi's sarcoma, the ^{111}In-labeled IgM may not be needed after initial testing.

***I.p. administration*:**

An indwelling catheter will be inserted transcutaneously. Free flow within the peritoneal cavity needs to be demonstrated with a radiographic contrast medium. If there are no serious hindrances to the dispersion of the contrast media, like adhesions, then the radioimmunoconjugate will be administered following the infusion of 1 to 1.5 l. of D5-1/4 NS (dextrose-saline solution).

For ^{111}In-labeled IgM, approximately 0.5 mg of IgM labeled with 1 mCi will be used.

For ^{90}Y-labeled IgM, the initial dose will be 1.0 mg of IgM labeled with 5 mCi. The next set of patients will be administered 1.0 mg of IgM labeled with 10 mCi. The last set of patients will be administered 2 x 1.0 mg of IgM labeled with 5 mCi. Other effective dosage ranges can be determined in the same manner using routine testing.

I.l. administration:

Depending on the site and size of the tumor, one or more injections will be made.

For the ^{111}In-labeled IgM, approximately 0.5 mg of IgM labeled with 0.3 mCi of ^{111}In will be injected.

For the ^{90}Y-labeled IgM, the initial activity will be approximately 0.01 mg of IgM labeled with 0.1 mCi of ^{90}Y per 1 cc^3 of tumor volume. The next activity to be tested will be 0.2 mCi per 1 cc^3 of tumor volume. If this is well tolerated then the final activity will be 0.4 mCi per 1 cc^3 of tumor volume. Other effective dosage ranges can be determined in the same manner using routine testing.

Treatment of Disorders other than Tumors

Although described herein primarily with reference to treatment of tumors or other types of overproliferation of benign tissue, such as endometriosis, the conjugates can also be administered for treatment of disorders induced by deposition of immune complexes, such as in RA, or overproliferation of tissue.

RA is a chronic inflammatory arthropathy of unknown cause, but with a suspected autoimmune component in its pathogenesis. RA is a serious invalidating and life shortening disease with a major socio-economic impact due to its high incidence and prevalence throughout the Western World. It is most common in women over 60 years of age, but can occur even in adolescents. Curative therapy is not available at this time. Chronic palliative therapies are used with serious iatrogenic side effects. For the painful inflammation of larger joints surgical intervention (synoviectomy) is ineffective and has been abandoned in most Centers.

In RA, synovial membrane proliferation is induced by lymphokines provoked by autoreactive immune cells in the tissues surrounding the joint. Radiation of the immune cells decreases lymphokine production by inducing lethal DNA damage in these cells and interrupts the synovial proliferation. Swelling and pain decreases in the joint affected with RA. Intra-articular instillation with colloidal Y-90 has been applied mainly in Europe with a 50% response rate of approximately two years duration. (DJMcCarthy and

WJKoopman Arthritis and Allied Conditions 12th edition. 1993, Lea & Febiger, Philadelphia) A number of papers have reported on the efficacy of treatment of RA by radiation induced synovectomy in animal models and human clinical studies. These studies have used colloidal chromic phosphate 32P, colloidal 198Au, rhenium 186, dysprosium-165, 90Y-silicate (see, for example, Spooren, et al., Eur. J. Nucl. Med. 10(9-10):441-445 (1985); Boerbooms, et al., Eur. J. Nucl. Med. 10(9-10):446-449 (1985); Zuckerman, et al., Int. J. Rad. Appl. Instrum [B] 14(3):211-218 (1987); Aguilera, et al., Rev. Med. Chil. 122(11):1283-1288 (1994)).

As used herein in a preferred embodiment for treatment of RA, normal human IgM (which can be obtained, for example, from Centra processing blood products, that usually discard this protein for lack of clinical use) is labelled with Y-90 and Y-90 labeled IgM instillations are used in place of colloidal Y-90. The advantages of the IgM are that it is a soluble product leading to better diffusion in the joint fluids and better dose distribution for all synovial membranes. The size of the IgM prevents it from entering blood vessels and leaving the intra-articular space. Some radioactive IgM might translocate to lymph nodes draining the joint due to the insertion of IgM in lymphvessels through the fenestrations common to those vessels. This might be of some additional therapeutic benefit if these nodes contain radiosensitive autoreactive lymphocytes. Toxicity is not anticipated. Dosing would be based on the volume of the intra-articular fluids and be 0.1 mCi per cc for initial studies. The initial patient population for study is large joint RA patients, Brooker grades 2-3. In summary the application would provide a cheap, out patient, patient friendly RA treatment.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Preparation of Therapeutic Radiolabeled anti-tumor IgMs.

Monoclonal Antibodies

A human monoclonal antibody secreted by the AC6C3 heterohybridoma was used in these experiments (Freedman, et al.,

Hybridoma 10:21-33 (1991); Chen, et al., Hum. Antibodies Hybridomas 5:131-142 (1994)). It is of the IgM isotype and is reactive with the cell membranes of human ovarian, breast and colon carcinomas, and certain other malignancies. At approximately annual intervals, the hybridoma is subjected to recloning and the secreted antibody tested for reactivity against an ovarian tumor cell line (SKOV3, American Type Culture Collection, Rockville, MD) by fluorescence-activated cell sorter (FACS) analysis. The experiments presented here were conducted with the antibody secreted by the recloned cell line, AC6C3-2B12, hereafter referred to as 2B12. An isotype matched human monoclonal IgM (CH-1B9) was utilized as an irrelevant control.

The human monoclonal IgM CR4E8 (Chen, et al. Hum. Antibodies Hybridomas 5:131-142 (1994)) recognizes a 55 kDa cell surface membrane protein found on a human cervical carcinoma cell line (SW756). It is reactive with squamous cell carcinomas of both cervical and head and neck origin along with breast and colon malignancies. An isotype matched human monoclonal IgM (CH-1B9) served as a specificity control.

Antibody Production

The hybridomas were cultured in either tissue culture flasks or spin culture flasks in Iscove's Modified Dulbecco's Medium (IMDM) with 20% v/v fetal bovine serum (FBS), 10% NCTC 109 (Life Technologies, Gaithersburg, MD), insulin (5 μ g/ml) and transferrin (5 μ g/ml). After the cells were at a suitable density and good viability, the growth media was removed by centrifuging the cultures in sterile centrifuge bottles. The media was poured off and then the cells were resuspended in the production media and placed back into sterile culture flasks. The production media contains only IMDM and insulin (5 μg/ml) so it is devoid of serum immunoglobulins that copurify with the monoclonal antibodies. After 3-4 days, when the viability dropped to 20-40%, the cell culture supernate was harvested by centrifuging the culture in sterile 500 ml centrifuge tubes. The supernate was poured off and saved and then sterile filtered using a 0.22 m filter. Sodium azide, 0.005% w/v, was added to the supernate which was then stored at 4°C.

Antibody Purification

The supernate (1-3 L) was concentrated on a CH2RS spiral cartridge concentrator (Amicon, Beverly, MA) equipped with 1 ft^2, 30 K or 100 K MW cutoff membrane. The volume of liquid was concentrated down to less than 200 ml by running the apparatus at a pump speed of 7-8 and a back pressure of 25-30 psi. Next 1 L of 0.1 M PBS, pH 7.3, with 1 mM EDTA was added and the solution reconcentrated back down to less than 200 ml. This was repeated with 0.1 M PBS and then the concentrated supernatant solution was collected. The filtration apparatus was then washed twice with approximately 200 ml of 0.1 M PBS which was collected.

The concentrated supernatant solution was then further concentrated using a 8200 stirred cell (Amicon, Beverly, MA) equipped with a 100 K MW cutoff membrane and charged with 10-15 psi of nitrogen gas. The solution was reduced to approximately 30 ml and then the first wash from above was added. The solution was reconcentrated and then the second wash was added. The solution was reconcentrated to approximately 25 ml and then collected. The apparatus was washed with 10 ml of PBS which was then added to the concentrated solution.

The solution at this point has been concentrated approximately 100-fold. The metal ions were removed through chelation with the EDTA and most of the proteins and other molecules below 100 kDa in size were filtered out. Large proteins were still present which were then removed by size-exclusion chromatography using a 2.5 x 100 cm column packed with Sephacryl S-300 HR (Pharmacia Biotech, Piscataway, NJ). Approximately 5-6 ml of the concentrated antibody solution was loaded on the column and the antibodies were eluted with 0.1 M PBS containing 0.001% NaN_3. Fractions were collected every 70 seconds starting at 160 min after elution commenced.

The IgM fraction eluted first and could usually be visually identified. Conformation of the peak was obtained by measuring the absorbance of the fractions at 280 nm. The integrity and the purity of the IgM fractions were checked by size-exclusion HPLC using a Bio-Silect SEC 250-5 column (Bio-Rad, Hercules, CA) with 0.2 M sodium phosphate buffer, pH 7.2, as the mobile phase. Fractions with a single peak that had a retention time equal to

that of a known IgM standard were kept. The fractions from several column purification runs were combined and the material was reanalyzed by HPLC. The purified IgM was stored at 4°C.

Fluorescence-Activated Cell Sorter Analysis

The reactivity of the immunoglobulins with the cell surface of their respective cell lines used in these experiments was demonstrated by FACS. A human ovarian carcinoma cell line (SKOV3), was used for 2B12. These cells were cultured in L15 media containing 10%(v/v) fetal bovine serum (FBS) in the presence of 5% CO_2 at 37°C. A human head and neck squamous cell cervical carcinoma cell line, 886, was obtained from Dr. Walter Hittleman (The University of Texas M. D. Anderson Cancer Center) (Sacks, et al., Int. J. Cancer 44:926-933 (1989)) and used with CR4E8. These cells were cultured in 1:1 mixture of Iscove's modified Dulbecco's media and McCoy's 5A media (Life Technologies, Gaithersburg, MD) containing 10% v/v FBS in the presence of 5% CO_2 at 37°C.

Cells were detached from the tissue culture flasks with Versene and washed with phosphate buffered saline (PBS) pH 7.3 containing 2% FBS and 0.02% NaN_3 (w/v). They were then incubated with 100 µl (100 µg) of human IgM for 60 min at 4°C, washed, and then incubated in a 1:100 dilution of FITC-conjugated goat antihuman $F(ab')_2$ for 30 min at 4°C. The cells were then washed and resuspended in PBS with 2% FCS and 0.02% NaN_3 followed by fixation in 2.0% (v/v) paraformaldehyde. Cell binding was examined with an EPICS profile analyzer (Coulter, Hialeah, FL).

Preparation of Immunoconjugates

To a solution of IgM (1×10^{-5} mmol) in 0.05 M Hepes buffer, pH 8.6, was added isothiocyanatobenzyl-3-methyl-diethylenetriamine pentaacetic acid (ITC-2B3M-DTPA) (1×10^{-4} mmol). The solution was gently swirled to dissolve the ITC-2B3M-DTPA. Then triethylamine, 1.5 M, pH 8.6 was added to the solution to yield a final concentration of the amine in the range of 7.5-10 mmol. The solution was then incubated at 4°C for 12 h. The immunoconjugate was purified from unreacted ITC-2B3M-DTPA by filtration in a Centricon-30 (Amicon Corp., Beverly, MA) and washed with 0.1 M PBS, pH 7.3. Purity was ascertained by size-exclusion HPLC using a

Bio-Silect SEC 250-5 column with 0.2 M sodium phosphate buffer, pH 7.2, as the mobile phase. The average number of DTPA molecules per IgM was then determined (Quadri, et al., J. Nucl. Med. 34:938-945 (1993)).

Radiolabeling of IgM-2B3M-DTPA Conjugates

^{111}In-Labeled Immunoconjugates

The ^{111}In-labeled immunoconjugates were prepared by mixing equal volumes of 0.6 M sodium acetate buffer, pH 5.3, with 0.06 M sodium citrate buffer, pH 5.5, and the immunoconjugate solution (10 mg/ml). Typically volumes of 100 µl were used. Next pure $^{111}InCl_3$ solution (typically 5 µ l, 1 mCi; New England Nuclear, Boston, MA) was added to the buffered immunoconjugate, mixed well, and allowed to incubate for 40 min. The radioimmunoconjugates were challenged with excess chelator by incubating the labeling mixture with 0.01 M diethylenetriamine pentaacetic acid (DTPA), pH 6.5, for 10 min. The radiolabeled IgM was purified on a Sephadex G50 gel column (1.5 x 20 cm) using 0.1 M PBS as the elutant. Fractions were collected and assayed with a CRC-15R dose calibrator (Capintec, Ramsey, NJ). The degree of radiometal incorporation and the purity of the radioimmunoconjugate was assessed at each step by instant thin-layer chromatography (ITLC) with saline as the mobile phase and thin-layer chromatography (TLC) using a 1:1 ratio of methanol and 10% (w/v) ammonium acetate in water as the mobile phase. The strips were cut in half and counted in a Cobra II gamma counter (Packard Instrument Co., Meriden, CT).

^{90}Y-Labeled Immunoconjugates

The ^{90}Y-labeled radioimmunoconjugates were prepared by mixing equal volumes of 2.0 M sodium acetate, pH 6.0, with the immunoconjugate solution (10 mg/ml). The combined volumes ranged from 100-200 l for the biodistribution studies to 300-600 µl for the therapeutic studies. To this was added pure $^{90}YCl_3$ solution (Battelle Pacific Northwest Laboratories, Richland, WA). Volumes and activities ranged from 1-5 µl, 2.0-5.0 mCi for the biodistribution studies and 10-20 µ l, 20-50 mCi for the therapeutic studies. The solution was mixed well and allowed to incubate for 60 min. The reaction was quenched with a hundred-fold excess of chelator using 0.01

M DTPA, pH 6.5. After a 10 min incubation, the solution was loaded onto a Sephadex G100 gel column (1.5 x 50 cm) and the radioimmunoconjugate eluted with 0.1 M PBS, pH 7.3. The fractions were collected and assayed for radioactivity using a dose calibrator. Purity was assessed at each step by ITLC with saline as the mobile phase and by TLC using a 1:4 ratio of 30% ammonium hydroxide and ethanol as the mobile phase.

All purified radioimmunoconjugate solutions were sterile filtered and then diluted to the appropriate activities using sterile 0.1 M PBS, pH 7.3. The radioimmunoconjugates had greater than 95% purity and were used within 2 h of their preparation.

Example 2: Administration of Radioisotope-labeled Antibodies into Tumors to Determine Biodistribution.

Materials and Methods

Radiolabelled Antibodies

Radiolabelled anti-tumor antibodies were prepared as described in Example 1.

The purified antibodies demonstrated cell-surface reactivity by FACS of 86% for CR4E8 versus 12% for CH-1B9. Size-exclusion HPLC analysis demonstrated a purity of 98% for the immunoconjugates after derivatization. An average of four 2B3M-DTPA molecules were conjugated to each immunoglobulin. Moreover, after purification of the radioimmunoconjugates at least 96% of the radioactivity was antibody bound and more than 90% of the radioactivity remained bound when incubated for up to 72 h at 37°C in human serum.

Preparation of ^{90}Y-Aggregate

Five microliters of YCl_3 (3.3-7.7 mCi) was mixed with 50 μ l of 0.05 N NaOH. The solution was vortexed vigorously. One hundred microliters of a 12.5% solution of human serum albumin in PBS, pH 7.3, was added. The mixture was vortexed again and then allowed to incubate for 1 h. A gelatinous substance formed. An aliquot of the aggregate was resuspended with sterile 0.1 M PBS, pH 7.3, for injection into mice. The suspension was checked by ITLC and TLC to ensure that the presence of radioactivity was limited to the aggregate phase. The ^{90}Y-aggregate preparations were greater than 99% pure and were administered within 1 h of their preparation.

Tumor Inoculation

I.P. Model

A human epithelial ovarian carcinoma cell line, SKOV3-NMP2 (Mujoo, et al., Oncogene 12:1617-1623 (1996)), was obtained from Dr. Kalpana Mujoo (The University of Texas M. D. Anderson Cancer Center). It was grown in MEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C. Cells were detached with 0.25% trypsin at 37°C for 2 min. The trypsin was neutralized with media, and the cells were then centrifuged at 800 x g. The cell pellet was then resuspended in media at the required cell density. Female athymic nude mice, 6-10 weeks old, received 0.2 ml of the cell suspension through i.p. administration using a 30-gauge needle. For the biodistribution, therapy and tumor burden experiments, mice received 2.5 x 10^6 cells. For the study of the effect of cell number on survival, mice received either 5 x 10^4, 5 x 10^5, 2.5 x 10^6 or 10^7 cells.

I.L. Model

The human head and neck squamous cell carcinoma cell line, 886, was cultured as above. Cells were detached with 0.1 % trypsin at 37°C for 2 minutes. The trypsin was neutralized with media and the cells were centrifuged at 800 x g. The cell pellet was resuspended in media to a density of 8 x 10^7 cells per ml. Male athymic nude mice, 6-10 weeks old, were injected with 0.1 ml of cell suspension subcutaneously using a 30-gauge needle.

The mice were housed in filter-top cages and provided with sterile food and water. Animal studies were conducted in compliance with the USDA

and Animal Welfare Act. Animal protocols were approved by the Animal Care and Use Committee at the M.D. Anderson Cancer Center.

Biodistribution Studies

I.P. Model

The reagents were administered to mice that received the tumor cells inoculum 12 days earlier. Both the i.p. and i.v. treated mice received approximately 200 μ l of the radioimmunoconjugate. The ^{111}In-treated mice received 10-15 μCi, except for the mice treated for the measurement of whole-body retention of radioactivity. They received 55 μCi. The ^{90}Y-IgM-treated mice received 40 μCi. The ^{90}Y-aggregate was administered i.p. to the mice. They received 30 μCi in approximately 200 μ l.

Mice treated i.p. were euthanized at 3, 24, 48, and 96 h post injection. Mice treated i.v. were euthanized at 24 and 48 h posttreatment. Blood was drawn by cardiac puncture and weighed. Normal tissue and tumor nodules were excised, rinsed in PBS, blotted dry, weighed, and then counted in a gamma counter along with the blood samples. The results were corrected for radioactive decay and were expressed as percent injected dose per gram of tissue (% ID/g).

I.L. Model

The reagents were administered to mice inoculated with tumor cells 4-5 weeks earlier. Tumors sizes ranged from 8-12 mm in diameter at the time of administration. All the i.l. treated mice received a single injection into the center of their tumor with approximately 10-20 μl of the radioimmunoconjugate. The i.v. treated mice received approximately 200 μl of the radioimmunoconjugate. The ^{111}In-IgM treated mice received approximately 5 -10 μCi of activity and the ^{90}Y-IgM treated mice received approximately 20 μCi of activity. The ^{90}Y-aggregate treated mice received approximately 5-10 μl at an activity range of 10-20 μCi.

Mice treated i.l. with a radioimmunoconjugate were euthanized at 3, 24, 48, 96 and 144 h post injection. Mice treated by i.v. administration were euthanized at 24 and 48 h posttreatment. Mice treated i.l. with ^{90}Y-aggregate were sacrificed at 3, 24, 48 and 96 h post administration. Blood was drawn

by cardiac puncture and weighed. Normal tissue and tumors were excised, rinsed in PBS, blotted, weighed, then counted in a gamma counter along with the blood samples. The uptake of radioactivity was expressed as % ID/g.

Therapy Studies

Intraperitoneal Therapy

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier, received unlabeled immunoconjugate (10 μg) in 200 μ l volume of 0.1M PBS i.p.

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier, received graded activities, 50, 100, 200, 300, 400 and 500 μCi, of ^{90}Y-labeled 2B12 in a volume of 200 μl. Ten animals were used for each activity. An additional 5 animals received 200 μCi in a volume of 1 ml. Mice were screened after four hours for retention of radioactivity by placing them inside a 50 ml centrifuge tube which was then placed inside a dose calibrator. Mice were assigned to the dose ranges indicated by the calibrator.

Nude mice inoculated intraperitoneally with 2.5 x 10^6 SKOV3 NMP2 cells twelve days earlier received either 75 μCi x 2, weekly; 100 μCi x 2, separated by 3 weeks; 165 μCi x 2, weekly; 170 μCi x 3, weekly; or 200 μCi x 2, separated by 3 weeks. The volume of the injectate was 200 μl. Mice were screened after each administration in the dose calibrator to ensure that they retained the administered radioactivity.

Intralesional Therapy

Nude mice bearing s.c. tumors 8-12 mm in diameter received i.l. 10 μg of the unlabeled immunoconjugate in 10 μl volume of 0.1 M PBS.

Nude mice bearing s.c. tumors 5-12 mm in diameter received graded activities, 20-1000 μCi, of the radioimmunoconjugate in a volume of 5-20 μl. Mice were placed in a dose calibrator after administration to check the level of activity retained in the tumors.

Nude mice bearing s.c. tumors 8-11 mm in diameter were injected with 100-400 μCi of the ^{90}Y-aggregate in a volume of 5-10 μl.

Calculation of Tumor Size

Tumor size was checked every 3-4 days. Tumor volumes were calculated by the formula:

$$\text{Volume} = \pi/6 \times \text{length} \times \text{width} \times \text{height}$$

The % ID/g of tumors for both ^{111}In-labeled CR4E8 (tumor-reactive) and ^{111}In-labeled CH-1B9 (irrelevant) antibodies were obtained from the i.l. biodistribution experiments and plotted with the % ID/g as the abscissa and time as the ordinate. The area under the curve for both radioimmunoconjugates was obtained by summation of the area of the trapezoids on the graphs.

Since measurement of tumor response in the intraperitoneal model was not possible without either performing surgery or sacrificing the animals, an indirect way of measuring tumor response was sought. It was reasoned that the number of surviving tumor cells in the mice might be obtained by comparing their mean survival time (S_{50}) to the mean survival time of untreated mice that were inoculated with different numbers of tumor cells. The number of cells needed to produce a given S_{50} can be found. This number divided by the initial injected standard number of cells, 2.5×10^6 in the treated mice provided the surviving fraction (SF). This demonstrates a log-linear relationship between the surviving fraction of cells in the treated mice and the administered activity. This survival curve lacks a quadratic function that is typically observed after high doses of high dose rate, sparsely ionizing radiation. The model commonly used to describe radiation survival curves produced by sparsely ionizing radiation is the linear-quadratic model (Chadwick, et al., Phys. Med. Biol. 18:78-87 (1973)). With this model,

$$SF = e^{-[\alpha D + \beta D^2]}$$

where D = dose, $e^{-[\alpha D]}$ = cell death by the linear component and $e^{-[\beta D2]}$ = cell death produced by the quadratic component. The original hypothesis of this radiation survival curve model postulated that the component described a single ionization track that produced a double strand break in nuclear DNA. While the component described two ionization tracks that each produced a single strand break. Two "beta" breaks occuring close together in space and

time can form a double strand break in DNA. Only double strand DNA breaks are thought to correlate with the lethal effects of radiation on cells. The linear-quadratic model predicts that x-rays and gamma rays (high dose rate but short duration) employed clinically will be more effective than RIT which lacks the component. The low dose rate of RIT allows for repair of single strand breaks. However RIT optimizes other modes of cell killing, such as accumulation of tumor cells in the more radiosensitive G_2 phase of the cell cycle (Marin, et al., Int. J. Radiat. Oncol. Biol. Phys. 21:397-402 (1991); Knowx, et al., Radiat. Res. 135:24-31 (1992)), accelerated dose delivery or increased tumor cell kill by a process described as protracted exposure sensitization (PES) (Williams, et al. Int. J. Radiat. Oncol. Biol. Phys. 24, 699-704 (1992)). These additional mechanisms of tumor cell kill make up for some of the tumor cell kill lost by the low initial dose rate of RIT. Only small differences in surviving fractions between equivalent doses of RIT and external beam radiation have been seen in experimental animal models (Fowler, et al., Int. J. Radiat. Oncol. Biol. Pphys. 1261-1269 (1990); Fowler, et al., Int. J. Radiat. Oncol. Biol. Phys. 18:1261-1269 (1990; Knox, Cancer Res. 50:4935-4950 (1990); Neacy, et al. J. Nucl. Med. 27:902-903 (1986)).

A drawback of the peritoneal carcinomatosis model is that tumor volume at the time of administration of the radioimmunoconjugate is not accurately known. A direct calculation of dose (energy deposited in a given volume) is therefore not possible. Indirect estimates of tumor doses from RIT can be made by extrapolation of the observed biologic responses. In the section on surviving fraction of tumor cells, calculations are given for determining the number of surviving tumor cells after i.p. RIT. In this section, another model used to describe the shape of a radiation survival curve, the multi-target single hit model (Johns, et al., The Physics of Radiology. 4th ed. Charles C. Thomas (Springfield, IL 1983) pp.679-681) will be employed to simplify the calculations for estimating the absorbed radiation dose from i.p. RIT. In the multi-target model,

$$SF = 1-(1-e^{-d/d_0})^n$$

where d = the initial slope of the survival curve, d_0 = the final slope, and n = the width of the shoulder of the curve (the extrapolation number). After the highest total amount of radioactivity administered i.p., tumor kill is estimated to be 3.5 logs. If external beam radiation and i.p. RIT show similar surviving fractions after similar doses of radiation and if one assumes an n of 3 and a D_0 of 2.0 Gy as radiation survival curve characteristics of SKOV3 NMP2:, the amount of external beam radiation needed in 2.0 Gy daily fractions to obtain 3.5 log tumor cell kill is approximately 52 Gy. However, the maximum tolerated dose to whole abdomen of human patients by external beam is less than 30 Gy. If the SKOV3 NMP2 cell line radiation survival curve parameters are chosen to reflect a more radiation sensitive tumor (n = 3, D_0 = 1.0 Gy), only 14 Gy of external beam radiation in 2 Gy fractions would be needed to reach an SF of 0.0005. Therefore, the current estimate for the dose delivered to tumor by RIT is between 3 and 10 Gy per 100 μCi of ^{90}Y (14-52 Gy divided by 500 μCi).

Autoradiography

Whole-Body

Two mice that had received i.p. ^{111}In-labeled 2B12 were sacrificed at 24 h postinjection. Their extremities were removed and the bodies frozen in 4% carboxymethyl cellulose. The frozen blocks were mounted on a cryomicrotome (Hacker Instruments, Fairfield, NJ), and sectioned into 50-m-thick coronal slices. Photographs were made of the sections, which were then mounted on tape and freeze dried. The sections were subsequently exposed to x-ray film (X-Omat AR, Kodak, Rochester, NY) for 48 h before development of images.

Tumor Autoradiography and Histology

Male nude mice bearing 8-12 mm s.c. tumors were injected with 5-10 μC of either ^{90}Y-labeled CR or ^{90}Y-aggregate in a volume of 5-10 μl. Mice were sacrificed at 3, 24, 48, 96 and 144 h. The tumors were excised, rinsed in PBS, blotted, covered with Tissue-Tek embedding medium (Miles Inc., Elkhart, IN) and then frozen at -20°C. The frozen blocks were later trimmed and mounted on cooled chucks in a refrigerated cryostat. Serial sections, 8

m thick, were taken approximately every 80 m through the entire tumor for the purpose of autoradiography. Adjacent sections, 4 m thick, were taken for histology. The sections were collected on slides. The histology specimens were stained with hematoxylin and eosin. The autoradiographic sections were mounted in sequence on stiff paper, covered with plastic film and then placed in a cassette with a phosphorous storage screen (Molecular Dynamics, Sunnyvale, CA). The screen was exposed for 1-2 days and then read on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Chords were drawn across the tumor images on the screen and counts were obtained along the length of the chords. Autoradiographs were obtained by placing slides on hyperfilm-ECL (Amersham, Arlington Heights, IL) for several hours to several days to expose the film.

Gamma Camera Imaging

Four mice that received i.l. ^{111}In-labeled CR4E8 were scanned at 24, 44, 68, 116 and 164 h postinjection. Five mice that received i.l. ^{111}In-labeled CH-1B9 were scanned at 24, 48, 96 and 144 h. Imaging was performed with a Starport gamma camera (General Electric Co., Milwaukee, WI) fitted with a medium energy collimator. The data was then transferred to a Triad gamma camera (Trionix, Cleveland OH). Regions of interest (ROI) were drawn over the tumors and counts were obtained for each time point. The counts per second were calculated and for the 2nd and subsequent time points, they were corrected for physical decay. The data was plotted as the percent remaining radioactivity in the ROI versus time. Biologic half-lives of the radioimmunoconjugates were then obtained from the graphs.

Results

Biodistribution of CR4E8 administered either i.l. or i.v.

The biodistribution data comparing i.l. and i.v. administration of ^{111}In-labeled CR4E8 showed that intralesional administration resulted in high tumor uptake at 24 h (108% ID/g) and the uptake remained elevated at 48 h (104% ID/g). The normal organs had low uptake, with kidney (3% ID/g) and liver (2% ID/g) being the highest at 24 h and both organs at 3% ID/g at 48 h. All other organs had <2% ID/g uptake at both time points. Blood radioactivity was <1% ID/g at both time points. In comparison, i.v.

administration resulted in very low tumor targeting (<1% ID/g) at both time points. Liver uptake was high (35% ID/g) and spleen uptake was moderate (≈ 14% ID/g) at both 24 and 48 h. Kidney had the next highest uptake (4% ID/g) at both time points. The blood clearance of radioactivity was nearly complete (1% ID/g) at 24 h.

Biodistribution data comparing the i.1. administration of CR4E8 labeled with ^{111}In or ^{90}Y showed that tumor deposition of radioactivity was high at 3 h (109% ID/g for ^{111}In and 102% ID/g for ^{90}Y, P=0.438) and remained elevated throughout the study with 56% ID/g for ^{111}In and 44% ID/g for ^{90}Y at 144 h (P=0.324). No statistically significant difference was found between the tumor deposition and retention of radioactivity for the radioimmunoconjugates. The normal organs demonstrated low uptake of radioactivity with both radiometals. Kidney (3% ID/g at 24 and 48 h for ^{111}In and 96 h for ^{90}Y) and liver (3% ID/g at 48 h for ^{111}In) had the highest uptake. Blood and bone radioactivity were <0.7% ID/g at all time points for both radiometals. Normal organs had similar levels of uptake of radioactivity for both radioimmunoconjugates at most time points with the exception of ^{111}In being >^{90}Y for kidney at 24 h (P=0.003) and ^{90}Y>^{111}In for femur at 96 h (P=0.005) and at 144 h (P=0.026).

Tumor deposition and retention of radioactivity for i.1.administered tumor-reactive and irrelevant IgM radiolabeled with ^{111}In shows that at 3 h tumor-reactive IgM had a higher deposition of radioactivity (109% ID/g) than irrelevant IgM (90% Id/g), but this was not statistically significant (P=0.324). At 144 h, irrelevant IgM showed moderate tumor retention (34% ID/g), while tumor-reactive IgM was retained more effectively (56% ID/g). This difference was statistically significant (P=0.021). The tumor uptake ratios of tumor-reactive to irrelevant IgM were 1.2 for 3 h, 1.3 for 24 h, 1.2 for 48 h, 1.7 for 96 h and 1.6 for 144 h. Normal organ uptake of irrelevant IgM was low with kidney having the highest value (2% ID/g) at 48 and 96 h. Blood radioactivity of irrelevant IgM was <1% ID/g at all time points.

Biodistribution data comparing the i.l. administration of ^{90}Y-labeled tumor-reactive IgM with ^{90}Y-aggregate show that tumor deposition and retention of radioactivity were higher throughout the course of the experiment with ^{90}Y-aggregate (126% ID/g at 3 h and 109% ID/g at 96 h) compared to ^{90}Y-labeled IgM (102% ID/g at 3 h and 77% ID/g at 96 h), but the sample size for ^{90}Y-aggregate was too small (n=3) to allow statistical analysis. With ^{90}Y-aggregate, bone had the highest uptake of radioactivity (3% ID/g) at 24 and 96 h, followed by kidney (1-2% ID/g). The other normal organs showed low uptake of radioactivity (<1% ID/g). Blood radioactivity was very low (<0.1% ID/g) at all time points.

Representative autoradiographs of sections from tumors treated with i.l. ^{90}Y-labeled CR4E8 or ^{90}Y-aggregate show that with ^{90}Y-labeled CR4E8 the majority of the radioactivity is localized at the injection site 3 h after administration. Additional radioactivity is apparent along the needle track and the periphery of the tumor. By 24 h, the radioactivity is widespread and remains so through the end of the experiment. In contrast, the ^{90}Y-aggregate remains localized at the injection site or along the needle track as discrete point sources of radioactivity for the entire experiment.

Activity levels along lines drawn across tumor images obtained at 48 h confirm that a greater volume of distribution of administered activity was achieved with the soluble radiolabeled IgM.

Images of a representative nude mouse injected 1.l. with ^{111}In-labeled tumor-reactive IgM show that the tumor deposition of radioactivity is high, with little uptake by the normal organs. The radioactivity remained localized and appears to become more homogenous within the tumor over time. The ROI analysis yielded tumor half-lives of 157 h for the tumor-reactive IgM and 96 h for the irrelevant IgM. Tumor half-lives determined by ROI analysis are similar to half-lives obtained in the biodistribution studies.

The whole-body retention of radioactivity in nude mice following the i.l. administration of ^{111}In-or ^{90}Y-labeled tumor-reactive IgM or ^{90}Y-aggregate shows that the elimination was monophasic for all reagents. Half-lives of approximately 73 h for ^{111}In-labeled IgM, 88 h for ^{90}Y-labeled IgM and 2200 h for ^{90}Y-aggregate were observed. The tumor half-lives of the

radioimmunoconjugates derived from the biodistribution experiments (143 h for ^{111}In-labeled IgM and 128 h for ^{90}Y-labeled IgM) were longer than the whole-body half-lives. In contrast, the tumor half-life of ^{90}Y-aggregate was shorter (475 h) than the whole-body half-life (2200 h).

Tumor Growth following i.l. Administration

When the unlabeled immunoconjugate was administered i.l., all mice showed continuous tumor growth like untreated mice and were euthanized when their tumors reached ≥15 mm in diameter. The "cold" immunoconjugate is not an effective immunotherapeutic agent. Metastasis (lymphatic or hematogenous) were not observed in this model.

When the radioimmunoconjugate was administered i.l, tumors were calculated to have received 80 Gy per 100 μCi of ^{90}Y-labeled IgM. The responses of animals treated with various activities of ^{90}Y-labeled CR4E8 is shown in Figure 1. All treated animals responded. As the majority of the tumors were rapidly ablated and tumor recurrences could not be linked to the original primary with absolute certainty, the results are expressed as the initial treatment volume and whether the tumor was ablated (complete response) or regressed (partial response). The graph demonstrates an apparent dose, but not dose/volume effect. Setting 150 μCi as an arbitrary cutoff point, the difference in tumor response above and below this level is statistical significance, $P = 0.001$. Tumor regression occurred with and without damage to the overlying skin. Tumor regression was marked by a color change in the tumor from pinkish white of viable tumor to a sallow, yellowish color.

Tumor ablation involved either crusting over and scabbing of the entire tumor or the tumor and surrounding normal skin were sloughed off resulting in an area of moist desquamation with a hole in the center where there once was tumor. Tumors frequently changed to a purplish pink color approximately 4-6 days after treatment prior to scabbing over. Other tumors turned whitish and necrosed from the center out. By 9-13 days, animals that received greater than 150 μCi to their tumor had a large area of moist desquamation that extended 2-4 mm beyond the margins of were the tumor was originally, as demonstrated by Figure 2. There were a few mice that had

extensive moist desquamation that appeared to result from leakage of the radioimmunoconjugate out of the tumor and into the surrounding subdermal tissue.

Fourteen out of 34 of the evaluable mice experienced tumor recurrence, as shown by Figure 3. Fewer mice are included in the tumor response duration analysis than in the analysis of tumor response and induction of moist desquamation due to the exclusion of animals with more than one single tumor nodule at the start of the experiment. This animal model relies on the subcutaneous inoculation of a cell suspension which may cause the formation of more than one tumor nodule in close proximity to each other. The biodistribution experiments demonstrated that both adjacent tumor masses and untreated masses on the opposite leg did not have significant uptake of radioactivity. Tumor recurrence were more frequent when less than 150 μCi was administered, $P = 0.039$. The majority of tumor recurrences occurred underneath the site of the original tumor. Tumor recurrence was less frequent in animals that showed moist desquamation.

When the ^{90}Y-Aggregate was administered i.l., all of the animals had a complete response. The morphologic changes that occurred in tumors after the ^{90}Y-aggregate treatment were markedly different from the changes in tumor after radioimmunoconjugate therapy. Tumors treated with high activities of ^{90}Y- aggregate were frequently intact at 9-14 days with central necrosis at the injection site. In contrast, tumors treated with the radioimmunoconjugate were sloughed off by this time point. Moist desquamation appeared later in the mice treated with ^{90}Y-aggregate and did not extend beyond the margin of the original palpable tumor.

Tumors recurred in 4 of the 8 mice treated with ^{90}Y-aggregate. In contrast, only 2 out of 12 of the mice treated with an equivalent activity of radiolabeled IgM had a tumor recurrence, $P = 0.06$.

Side Effects following i.l. Administration

No obvious, systemic toxicity was observed in any of the mice treated with ^{90}Y-labeled CR4E8, including mice that received 400-1000 μCi. Based on this fact and the very low level of radioactivity found in normal organs in the biodistribution studies, the weight of the mice was not measured. The

biodistribution data showed bone uptake and long whole-body retention of ^{90}Y in mice treated with ^{90}Y-aggregate, so their weight was monitored. They maintained their weight within 2% of their pretreatment weight signifying that no overt systemic toxicity was associated with this therapy.

Significant moist desquamation was observed in 13 out of 13 mice treated with > 150 μCi of the radioimmunoconjugate while mice treated with less than 150 μCi had moist desquamation in 6 out of 22 mice. This difference is statistically significant, $P = 0.00002$. Mice treated with the ^{90}Y-aggregate had moist desquamation of a reduced extent compared to mice treated with similar activities of the radioimmunoconjugate.

Blood counts in tumor-bearing mice following treatment with a single dose of ^{90}Y-labeled CR4E8 (average activity 308 μCi) were measured. On day 19 posttreatment, segmented neutrophils dropped to 40% and lymphocytes to 21% of their initial values. The RBC count was 92% of the initial value. On day 40 posttreatment, segmented neutrophils and lymphocytes were both at 58% of their initial value. The RBC count was 90% of the initial value.

Blood counts in tumor-bearing mice following treatment with a single dose of ^{90}Y-aggregate (average activity 287 μCi) were measured. On day 21 posttreatment, segmented neutrophils were at 71% and lymphocytes were at 40% of their initial values. The RBC count was stable at 103% of the initial value. On day 44 posttreatment, segmented neutrophils continued to drop to 51% of the initial value. Lymphocytes had recovered and were at 98% of the pretreatment value. The RBC count was at 101% of the initial value.

Mice treated with low activities (≤ 100 μCi) of the radioimmunoconjugate had mild skin damage in the form of reduced or absent adnexa (hair follicles, sebaceous and sweat glands). Some mice treated with higher activities had in addition to reduced or absent adnexa, fibrosis of the dermis, subcutis and skeletal muscle, vasculopathies and necrosis of skeletal muscle. The absence of skin adnexa is not indicative of radiation damage and is probably due to secondary healing of the denuded areas. The fibrosis and vasculopathies were attributed to radiation injury.

Tumor Growth Following Intraperitoneal Administration

The intraperitoneal administration of radioimmunoconjugate but not unlabeled immunoconjugate was able to prolong the lives of tumor bearing mice. In fact, there is a strong positive correlation between the amount of radioactivity administered and the prolongation of survival. For each 100 μCi of activity, from 0-300 μCi, the survival increased an average of 12 days. With high activities (300 μCi and above), early death occurred 12-14 days posttreatment.

Fractionated administration of radioimmunoconjugate also prolonged the lives of treated mice in a dose-responsive manner if the fractions were administered within an appropriate time period. For mice receiving a low activity (75-100 μCi) per fraction, the fractions could not be administered more than three weeks apart. With medium levels of activity (165-200 μCi) per fraction, the interval between fractions could be between 1-3 weeks. Mice treated in this manner demonstrated an increase in survival of 13 days for every 100 μCi of activity administered from 150 to 510 μCi. Fractionation increased the total activity that could be administered without producing weight loss or early death while at the same time maintaining the therapeutic efficacy.

High activities (300 μCi and above) administered as a single dose produced overt toxicity. Both weight loss in excess of 10% of initial body weight and early death were noted in groups treated at such doses. In contrast, fractionated administration did not cause overt toxicity even up to 510 Ci.

Blood counts were obtained from mice treated with an average of 133 Ci as a single dose and from mice that received 75 μCi x 2, separated by 2 weeks. Approximately 4 weeks from the initiation of treatment, both sets of mice demonstrated an increase in their segmented neutrophils. Mice treated with a single dose had more than twice as many segmented neutrophils than the fractionated mice. Lymphocytes in both groups of mice dropped to similar levels, 28% of the initial value.

The drop in peripheral blood lymphocyte count was expected as lymphocytes are known to be radiosensitive and to die rapidly from apoptosis after receiving low doses of radiation (Anderson, et al., Adv. Immunol. 24:215-335 (1976)). The increase in segmented neutrophils in the peripheral blood on the other hand was unexpected and may best be explained by the secretion of a growth factor specific for segmented neutrophils by the tumor cells or other cells in the peritoneal cavity. Indeed, various growth factors have been found in malignant human ascitic fluid (Hirte, et al., Proc. Ann. Meet. Am. Assoc. Cancer Res. 35:A258 (Abstract) (1994); Hirte, et al., Proc. Ann. Meet. Am. Assoc. Cancer Res. 34:A1428 (Abstract) (1993)), and growth factors specific for segmented neutrophils have been isolated and cloned: granulocyte-colony stimulating factor (G-CSF) (Nagata, et al., Nature 319:415-418 (1986)) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Wong, et al., Sciences 228:810-815 (1985)).

To confirm that the increase in segmented neutrophils was due to the tumor burden of the mice and not a compensatory mechanism in response to irradiation, mice from the tumor burden study had their blood sampled at different time points. There was an increase in the segmented neutrophil count over time. In one mouse that fortuitously did not have any tumor deposits, no increase in segmented neutrophils was observed.

The different responses noted for lymphocytes, granulocytes and erythrocytes after tumor inoculation and subsequent RIT indicate that the tumor has specific effects on the granulocytic line of hemopoiesis and not on hemopoietic stem cells. Similarly, i.p. RIT does not depress erythrocytes or lymphocytes to the same degree as granulocytes. Again, this indicates that decreased tumor burden leads to less granulocyte growth factor production and that relatively little stem cell damage was inflicted by i.p. RIT.

Discussion

The primary goal of this study was to test the concept of intracompartmental RIT with human monoclonal tumor-reactive IgM. This was accomplished through the use of 2 nude mouse models of human cancer and 2 locoregional modes of administration: i.p. and i.l. Both routes of

administration were compared against i.v. administration. Both the i.p. and i.l. routes demonstrated high, selective tumor deposition of radioactivity with lower normal tissue radioactivity than observed after i.v. administration.

When therapeutic activities were explored, i.p. administration was able to kill at least 3 logs of aggressively growing tumor without overt toxicity. Intralesional RIT produced complete tumor responses of long duration with minimal normal tissue toxicity. All therapeutic responses were due to radiation as the unlabeled "cold" immunoconjugate had no therapeutic effects.

Intralesional ^{90}Y-labeled CR4E8 was an effective therapeutic agent in a single administration. The only significant acute normal tissue side effect noticed was moist desquamation of the skin overlying and surrounding the tumor. Fractionation experiments to decrease this toxicity were not performed.

The ^{90}Y-aggregate remained localized in the center of the tumor nodule and was a less effective therapeutic agent. It also caused less moist desquamation presumably due to the larger distance between the radioisotope and the skin, leading to a lower radiation dose to skin.

This preclinical study demonstrates that i.l. but not i.v. administration of radiolabeled human tumor-reactive IgM results in high tumor radioactivity. Intralesionally administered radiolabeled IgM remained localized in the tumor, with low levels of radioactivity in the blood and subsequently low uptake of radioactivity in normal organs. In comparison, i.v. administered radiolabeled IgM was rapidly cleared from the blood with high liver and moderate spleen uptake. Intravenous administration of radiolabeled human IgM produced a similar biodistribution in other mouse models [Abeleve, Adv. Cancer Res. 14:295-350 (1971); Markoe, et al., Front Radiat. Ther. Oncol. 24:214-224 (1990) and may demonstrate significant liver uptake in human (Mach, et al. Cancer Res. 43:5593-5600 (1983)).

Intralesionally administered tumor-reactive IgM labeled with ^{111}In or ^{90}Y demonstrated similar high tumor uptake and retention. Normal tissue uptake of radioactivity was low, with kidney and liver having the highest uptakes (between 1 and 3% ID/g). Kidney activity may have been related

either to the metabolism of chelated light chains (Goldenberg, et al., J. Clin. Oncol. 9:548-564 (1991)) or to the urinary elimination of radioactive low-molecular-weight species; liver activity may have resulted from uptake of intact immunoglobulin from the blood.

Blood radioactivity was less than 0.7% ID/g for the tumor-reactive radioimmunoconjugates at all time points; this suggests that myelotoxicity will not be the dose-limiting normal tissue toxicity. The dose-limiting normal tissue, which remains to be defined, will probably be in the vicinity of the treated tumor. This tissue may vary, depending upon the location of the tumor and it may include vasculature, nervous, or interstitial tissue.

Bone radioactivity was less than 0.7% ID/g for the tumor-reactive radioimmunoconjugates at all time point. The clearance of radioactivity from bone was faster for ^{111}In-labeled IgM. This may account for the higher kidney activity seen for ^{111}In-labeled IgM at both 24 and 48 h. A small amount of ^{90}Y incorporated into bone could explain the slower bone clearance of ^{90}Y. The low level of bone radioactivity (0.5-0.6% ID/g) is not expected tc have significant biologic effects (Lewis, et al., Hybridoma 14:115-120 (1995)).

The long biologic half-lives of the radioimmunoconjugates in tumor (4–6 days) indicates that greater effective half-lives of the radioimmunoconjugates and hence radiation doses could be achieved through the use of therapeutic radioisotopes with half-lives longer than ^{90}Y (2.7 days). With such isotopes, the observed difference in tumor retention between tumor-reactive and irrelevant IgM, which became statistically significant at 144 h, would be of greater therapeutic significance.

The i.1. administration of ^{90}Y-aggregate produced high tumor deposition of radioactivity which remained elevated longer than ^{90}Y-labeled tumor-reactive IgM and resulted in a biologic half-life of 20 days in tumor. While this longer half-life suggests that higher radiation doses to tumor could be administered with ^{90}Y-aggregate, comparison of whole-body half-lives with tumor half-lives and examination of tumor autoradiographs predict that overall 90Y-labeled IgM will be the superior therapeutic agent.

The different tumor and whole body half-lives of the radioimmunoconjugates compared to the ^{90}Y-aggregate suggest fundamental differences in the metabolism of these reagents. The tumor half-lives of the radioimmunoconjugates are longer than their whole-body half-lives. Radioactive fragments produced from the metabolism of the radioimmunoconjugates appear to be rapidly eliminated from the body. This is in contrast to what is observed with the ^{90}Y-aggregate where free ^{90}Y from ^{90}Y-aggregate appears to be retained in the body by transchelation to the bone matrix.

Autoradiographs from sectional tumors demonstrate that tumor-reactive IgM diffuses throughout the tumor, whereas ^{90}Y-aggregate remains localized at the injection site. Analysis of the distribution of the counts per pixel across lines drawn on the tumor images confirmed the greater distribution of radioactivity with the radioimmunoconjugate. Thus, the radioimmunoconjugate provides a more uniform dose distribution within the tumor than the ^{90}Y-aggregate. The combined advantages of more uniform dose distribution within the tumor and rapid elimination of metabolized radioimmunoconjugate should provide a therapeutic advantage to the radioimmunoconjugate.

Serial gamma camera imaging corroborated the results of the biodistribution studies. The images show that high initial tumor deposition of radioactivity is achieved with i.l. administration of radiolabeled IgM and that the radioactivity remains localized. Furthermore, the activity appeared to become more homogenous over time. Very little normal tissue uptake of radioactivity was evident with either tumor-reactive or irrelevant radiolabeled IgM. Tumor half-lives calculated by ROI analysis agree with those obtained in biodistribution experiments. Together, these results suggest that the serial imaging of a small group of animals with a gamma camera can replace the more animal-intensive biodistribution experiments in the initial screening of radioimmunoconjugates for local administration.

Example 3: Protocol for Intraperitoneal IgM for the treatment of peritoneal carcinomatosis.

Patients with peritoneal carcinomatosis in general have one of the four primaries mentioned in this study: ovary, colon, pancreas, breast. Currently available treatment is unsatisfactory and median survival time is approximately three months.

Abstract

Protocol:

Patient Eligibility:

1. Histologically or cytologically confirmed epithelial ovarian carcinoma with extra ovarian peritoneal involvement - colon, breast, or pancreatic cancer in the peritoneal cavity.
2. Measurable disease.
3. Absence of small bowel obstruction.
4. Zubrod 0, 1, 2. Age ≥18 years. Negative pregnancy test.
5. Signed informed consent.

Treatment Plan:

1. Place temporary intraperitoneal catheter.
2. Verify free flow by radiographic contrast.
3. Instill 1-1.5 LD5 - 1/4 NS.
4. Obtain peritoneal fluid, blood and urine samples for immunoperoxidase staining and radiopharmacokinetic baseline studies.
5. Administer 1 mCi ^{111}In labeled AC6C3-H8 IP.
6. Sample peritoneal fluid, blood, urine 2, 20, 40, 72, and 150 hrs.
7. Whole body gamma scans at 2, 20, 40, 72 and 150 hrs.
8. SPECT at 41 hours of the abdomen.
9. Administer 5, 10 or 3x5 mCi of ^{90}Y labeled AC6C3-H8 IP after repeating steps 1 59 3.

Patient Evaluation:

1. Complete history and physical.
2. CBC with differential, platelets, PT/PTT.

3. Liver functions to include SGOT, SGPT, alkaline phosphatase and bilirubin.
4. Renal function tests including BUN, creatinine and urinalysis.
5. Abdominal and pelvic CT±contrast, chest x-ray and any other tests needed for tumor documentation.
6. 5cc serum for immunologic studies.
7. Review of all hospital records and pathology reports. Pathologic reviews of available specimens to confirm the diagnosis.

Statistical Considerations:

The main objectives of this study are to establish safety and to obtain information on potential utility. Data to be obtained from this study may be used in the design of future trails on a larger number of patients. The pilot trial will be stopped if tumor localization is not shown in the first 6 patients or if grade III or higher toxicity occurs in equal or more than one-third of patients. The first 6 patients will receive 5 mCi of ^{90}Y, the next 6 patients will receive 10 mCi of ^{90}Y, the last 6 patients will receive 5 mCi of ^{90}Y 3 times at 1 week intervals.

Reagents:

Antibodies

AC6C3-H8 is a new human tumor cell surface reactive monoclonal antibody (HMAB) obtained by fusing lymph node lymphocytes from a patient with epithelial ovarian carcinoma (EOC) with cells of the SPAZ 4 heterohybridoma line (Freedman, et al., Hybridoma 10:21-33 (1991)). This antibody is an IgM2 and recognizes a determinant expressed on the cell surface of an ovarian carcinoma cell line that can be detected by fluorescent activated cell sorter analysis (FACS) (Quadri, et al., J. Nucl. Med. 34:938-945 (1993)). The AC6C3 does not stain nylon wool purified nonadherent peripheral blood lymphocytes or red blood cells from normal donors. AC6C3 stains nonpermeabilized tissue sections of ovarian carcinoma and ovarian tumor xenografts using indirect immunoperoxidase. Most normal tissues including brain, liver, heart and kidney give negative or weak reactions to AC6C3. The antibody also reacts with other adenocarcinomas including breast and colon and may therefore be described as "pan-reactive".

The antibody mediates complement-dependent cytotoxicity and precipitates a 32Kd band by Western blotting immonoprecipitation experiments conducted on $SKOV_3$ cultured ovarian cancer cells. The association constant for AC6C3 is 2.3 x $10^{10}M^{-1}$ which was determined by saturation binding studies (Quadri, et al. 1993). AC6C3 may have certain advantages in clinical trials in patients with peritoneal carcinomatosis: (i) The human mAb may be used in repeated dosing schedules assuming anti-human antibodies are not produced. (ii) Although IgG mAbs may be preferred for clinical trials, IgMs may also be attractive for intracavitary treatments because of their large size and likelihood of a prolonged residence time. Prolonged residence time of the mAb in the peritoneal cavity may promote greater concentrations of the mAb on the tumor cells. AC6C3-H8 is a novel entity with a potential role for in vivo diagnosis and therapy of ovarian carcinoma.

Purification of Human Monoclonal IgM Antibody (HuMoAb) from Culture Media:

The media supernatant was concentrated by using Amicon Stirred Cell unit mounted with PM30 membrane at 4°C in a cold room. The retentate containing IgM was purified on Sephacryl S-300 column (2.5 x 50cm) to isolate the pure IgM fraction. The column was eluted with 0.05 M PBS pH 7.4 at a flow rate of 1.5 ml per minute. Purity of IgM was analyze by size-exclusion HPLC using two gel filtration columns (GF 250 & GF 450; 25 x 0.94 cm i.d.) connected in tandem, and SDS-PAGE techniques. Protein concentration was measured by UV spectrophotometer at 280nm absorbence. The IgM protein was sterile filtered through 0.2 μm Acrodisc and stored in aliquots of 4.0 mg/ml in PBS at 4°C in sterile tubes.

Conjugation of Antibodies

Human IgM AC6C3-H8 was purified and conjugated with an average of two ITCB-DTPA chelating agents per IgM molecule (Leichner, et al., Antibody Immunoconjugates and Radiopharmaceuticals 2:125-144 (1989)). The purified immunoconjugates showed no loss of activity to the ovarian carcinoma cell line ($SKOV_3$) *in vitro*. The ^{111}In labeled immunoconjugates were injected intravenously into nude mice bearing a human ovarian carcinoma ($SKOV_3$) xenograft. The human IgM conjugate cleared rapidly

from the circulation with a T1/2 (half-life) in the blood of less than 10 hours. Most of the activity was retained in the liver because of the large molecular size of the IgM molecule. The tumor targeting peaked at 48 hours. the tumor-to-muscle and tumor-to-blood ratios were 4:1 and 2:1 respectively at day 6. The immunoscintigraphy study demonstrated that radioactivity peaked throughout the tumor mass at 2 days post IgM administration. The tumor uptake in the biodistribution study showed 4-6% injected dose/gram of tumor at 48 hrs (n=3). Direct intraperitoneal administration of AC6C3-H8 to nude mice with higher tumor radioactivity (60% of injected dose/g of tumor) - therapy with Y^{90} labeled IgM prolonged survival of mice with extensive peritoneal carcinomatosis three-fold. In mice, fractionated therapy (once per week) is more effective and less toxic than single fraction therapy.

Preparation of Immunoconjugate:

The chelator, isothiocyanatobenzyl-DTPA derivative (ITCB-DTPA), is synthesized by previously published techniques. ITCB-DTPA was reacted with amino group of lysine residue of antibody to conjugate through a thiourea linkage. Antibody (9.0 mg, 1×10^{-5} mmole) in 0.05 M Hepes buffer pH 7.6 was reacted with freshly prepared 100 µl aqueous solution of ITCB-DTPA (60 µg, 1×10^{-4} mmole) at a molar ratio of 1:10. The pH of the reaction mixture was adjusted to 7.6 by 0.2 m $NaHCO_3$ solution and mixture was incubated for two hr at room temperature. Two DTPA ligands were conjugated per antibody molecule. Prior to radiolabeling, MoAb-thiourea-DTPA conjugate was purified from unconjugated DTPA by dialysis against PBS.

Radiolabeling of Immunoconjugate:

An aliquot (100 µl) of pure $^{111}In\ Cl_3$ (8.0 mCi) in 0.05 N HC1 is equilibrated with 100 l of 0.5 M acetate buffer pH 5.3 and 100 µl of 0.05 M citrate buffer pH 5.5. Two hundred microliters of immunoconjugate (2.0) mg) solution in PBS is added into buffered indium, mixed well, and incubated at room temperature for 30 min. The labeled immunoconjugate is separated from low molecular weight compounds by Sephadex G50 gel column (1.5 x 20 cm) chromatography using 0.05 M PBS as an eluent. The labeled immunoconjugage is collected and assayed in a dose calibrator, and

the labeling efficiency is determined by ITLC. The percentage radioactivity bound to the antibody is determined by TLC and HPLC analysis. The procedure for ^{90}Y Cl_3 labeling is similar, with the exception of different buffer with slightly higher pH and a G100 column instead of a G-50.

Quality Control Analysis:

Instant Thin Layer Chromatography (ITLC)

Radiolabeling yield of immunoconjugate is determined by ITLC using saline as developing solvent. Radioimmunoconjugate remains at origin while labeled DTPA moves with the solvent front. The ITLC strip is cut into three portions and counted on a gamma counter.

Thin Layer Chromatography (TLC)

Silica-gel TLC strip (1 x 12) is spotted with radiolabeled conjugate and developed by using methanol:10% NH_4OAc (1:1) as a mobile phase. In this TLC analysis, radiolabeled antibody appeared at R_f=0 and indium labeled DTPA/citrate at R_f=1.0. TLC strips are monitored by a radio chromatography scanner. the percent activity bound to the antibody fraction is determined by counting TLC strip in a gamma counter.

HPLC Analysis

Size exclusion HPLC analysis with a Bio-Sil SEC 250 column (300 x 7.8 mm i.d.) is used to determined the molecular weight and purity of radioimmunoconjugates.

The column was eluted with buffer (containing 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.9) at a flow rate of 1 ml/min.

Sterility and LAL Assay

The radioimmunoconjugate in PBS solution is filtered through 0.2 micron Acrodisc filter and tested for lack of pyrogens by Limulus Amebocyte Lysate assay (LAL). A small aliquot of the antibody batch (0.25 ml) is diluted to a final volume of 5 ml with 0.9% sodium chloride and tested for sterility using the protocol outlined by the FDA Code of Federal Regulation CFR 21, Part 210. Two ml aliquots are inoculated into fluid thioglycollate medium and soybean casein digest. The sample is incubated at 30-35°C, respectively for a period of 14 days. The aliquots are checked visually for bacterial growth. The pyrogen test is performed before the

administration of radiolabeled antibody, whereas the sterility test is completed for the whole batch only, not for individual patient administration.

Drug Composition

Indium-111 labeled human monoclonal IgM-DTPA conjugate in 5.0 ml PBS solution. The drug contains the following components:

Human Monoclonal IgM-DTPA	2.0 mg
Chelated Indium-111	5.0 mCi
0.05 M Phosphate Buffer Saline	5.0 ml
Human Serum Albumin (0.1%)	5.0 mg

Patients:

Patient Elgibility

Histologically or cytologically confirmed epithelial ovarian carcinoma, colon, breast or pancreatic carcinoma.

Patients with total or subtotal small bowel obstruction are not eligible.

Documentation of peritoneal carcinomatosis by clinical documentation, prior surgery, computerized tomography (CT) or magnetic resonance imaging (MRI).

Karnofsky ≥60, ECOG 0, 1 or 2.

Age ≥18 years.

Negative pregnancy test in women at risk.

No untreated active infection

Absolute granulocytes above 1000/mm^3 and platelets above 50,000/mm^3.

Pre-study Evaluation

Complete history and physical including height and weight documentation of signs and symptoms and performance status will be done in the week before the study commences (Appendix B).

Laboratory and Radiologic Studies

CBC with differential, platelets, PT/PTT.

Liver functions to include SGPT, alkaline phosphatase and bilirubin.

Renal function tests including BUN, creatinine and urinalysis.

Abdominal and pelvic CT ± contrast, chest x-ray and any other tests needed for tumor documentation.

5 cc serum as baseline for future F/U studies.

Review of all hospital records and pathology reports. Pathologic reviews of available specimens in studies to confirm diagnosis.

Evaluation During Study

Pharmacokinetics, tumor targeting and associations of uptake with clinical or radiological findings will be done as described below.

Blood, peritoneal fluid, urine sample, whole body gamma camera scan at 2 hours. Patient samples obtained and sampled under radiation safety precautions in Radiotherapy. Scan to be performed in Nuclear Medicine.

Repeat studies described in 6.3.1 at 20,40,72 and 150 hours. Scans will for peritoneum, and blood half-life by region of interest (ROI) analysis. Tissue distribution will be evaluated on scans as well. Inhomogeneities in radioimmunoconjugate distribution will be evaluated on SPECT (at 40 hours). Comparisons will be made with tumor deposits observed on CT. Tumor dosimetry will be done following the University of Nebraska protocol (Drs. Leichner, Hawkins, Akabani) (Hawkins, et al. IEEE Trans. Med. Imag. 7:135-148 (1988)) as soon as the required software is operational in the Arlington Cancer Center.

Recent radiologic findings (within 4 weeks) will be correlated with results of scanning.

Treatment:

Discussion of protocol and signed informed consent before entry.

A temporary intraperitoneal catheter will be inserted transcutaneously.

Verify free flow by injecting approximately 50 cc radiographic contrast IP. Under fluoroscopy orthogonal x-rays will be taken for documentation.

Infuse 1-1.5L D5-1/4 NS warmed to 37°C IP.

One hundred cc fluid removed for immunoperoxidase staining of tumor cells with biotinylated peroxidase conjugated HuMoAb and irrelevant HuMoAb and baseline for studies listed under t.6.

Administer 1 mCi ^{111}In labeled AC6C3-H8 IP in approximately 10cc. The antibody will be administered under the supervision of a physician.

Vital signs q 15 minutes for 2 hours. Patient in decubitis, anti-decubitis, left lateral and right lateral positions, prone and supine positions for 20 minutes each.

Five, ten or 3x five mCi of ^{90}Y labeled AC6C3-H8 will be administered following identical methods as described for the 111In labeled product. ^{90}Y labeled IgM will only be administered if the ^{111}In labeled IgM stays confined to the peritoneal cavity ()75% of injected dose) and tumor targeting is observed.

Dose of MoAb:

Starting dose will be 0.5 mg. If no, or poor, imaging is observed in first six patients next dose level will be 5 mg.

Evaluation and Management of Toxicity:

If 2 or more of the first 6 patients in either group develop grade III or IV toxicity, the protocol will be stopped. CBC and SMAC will be repeated weekly for the first 8 weeks after each administration and once a month thereafter, scans will be repeated at 2-month intervals.

Study End-Points:

Pharmacokinetics:

Compartmental and noncompartmental pharmacokinetic techniques will be used to determine the time area under the concentration curve, peak concentration, time to peak concentration, mean residence time distribution volume, clearance, intraperitoneal labeled antibody concentrations.

Immunotargeting:

Uptake and elimination of the radioimmunoconjugate will be studied for: (1) peritoneal cavity; (2) tumor nodules; (3) blood; (4) liver and spleen; (5) kidney; (6) urine. *In vivo* decay of radioactivity can be determined by planar views of the gamma camera. Tumor volumes and radioactive content of tumors and smaller volume normal tissues will require SPECT. The ideal radioimmunoconjugate will target tumor, have a long residence time in the peritoneal cavity with little or no spill-over into the blood. Normal tissue uptake should be low. The data points to be obtained will allow for

calculating the relative biodistribution of the radioimmunoconjugate over time in the listed compartments.

Tumor Dosimetry

Cumulative radioactivity in tumor volumes will be calculated by area under the curve techniques. Translation/extrapolation from Indium radioactivity to Yttrium dosimetry will be made for the planned administration of 5, 10 or 3 x 5 mCi of Yttrium labeled radioimmunoconjugate per kilogram, retrospectively when the appropriate computer programs have been installed and verified.

Criteria for Removal from Study:

Noncompliance or inability to complete follow-up studies. Grade III toxicity or tumor progression.

Example 4: Protocol for Intralesional Radiolabeled Antiferritin IgM of recurrent or persistent Hodgkin's Disease, Kaposi Sarcoma, and Head & Neck Cancer.

Approximately 7000 new cases of Hodgkin's disease (HD) are diagnosed each year in the United States. Initial treatment with chemotherapy, radiation or a combination of the two is successful in inducing a response in the vast majority of patients. Nonetheless approximately 30-40% of patients will relapse or fail to achieve an initial remission. Second line treatment with salvage chemotherapy is curative in a small group of patients. Second or third line treatment with high dose chemotherapy followed by autologous bone marrow transplantation may be curative in certain patients. Patients who fail second or "higher" line treatment and those who are not eligible for bone marrow transplantation are rarely cured and are candidates for Phase I studies. Phase I-II studies with ^{90}Y labeled polyclonal antiferritin have been completed. It was found to be an active single agent for relapsed Hodgkin's Disease with response rate of approximately 65% in a heavily pretreated poor prognosis group of patients. In a proportion of patients a single large mass (e.g. mediastinum) is a persistent problem after extensive prior chemo/radiation therapy.

Kaposi's Sarcoma (KS) is a vascular tumor arising from the mid-dermis. It consists of interweaving bands of spindle cells and irregular slit-

like vascular channels embedded in reticular and collagen fibers, infiltrations with mononuclear cells, and plasma cells. It was first described by the Austrian dermatologist Kaposi. Kaposi's Sarcoma (KS) occurs frequently in immunodeficient patients, such as HIV positive patients or allograft recipients. The skin, oral mucosa, lymph nodes and visceral organs such as the submucosa of the gastrointestinal tract, lung, liver and spleen may be involved. Systemic therapy for Kaposi's Sarcoma is poorly tolerated due to preexisting immunodeficiency or myelo-suppression. KS tumors are considered radiosensitive and palliation can be obtained with doses as low as 16 Gy, External beam radiation can be toxic in KS patients, especially in terms of severe radiation induced mucositis. All tested KS lesions contain ferritin (N=40).

The combination of tobacco and alcohol use are the best established and most significant carcinogens of mucosal malignancies in the head and neck area. The majority of patients have squamous cell carcinoma (HNSCCa) and present with large (> 6 cm) primaries and/or large lymph nodes metastases. Combination of radical surgery and high dose external beam radiation can control the disease in a proportion of the patients. However disease recurs in at least 30% of patients, who then become candidates for phase I chemotherapy. Distant metastases are relatively uncommon in such patients and the use of intralesional IgM could provide a new treatment option for such patients after prior high dose radiation. Up to 50% of squamous cell carcinoma's of the Head and Neck appear to contain significant amounts of ferritin.

Abstract

Protocol:

Patients:

Histological proof of recurrent or persistent Hodgkin's Disease, Kaposi Sarcoma, and Head & Neck Cancer.

Patients must be >10 years of age.

Patients must have measurable disease.

Treatment Plan:

0.3 mCi of indium-111 labeled antiferritin is deposited in the tumor mass. Whole body gamma camera imaging will be performed at 1, 20, 40, 120, and 165 hours after administration. SPECT scans will be performed of tumor containing areas at 40 and 165 hours after administration. Blood and urine samples will be taken at the same time points as for whole body gamma camera imaging. All urine will be collected for the first 48 hour after administration.

Patients will receive 0.1 mCi per gram of tumor of yttrium-90 labeled antiferritin which will be divided in three equal volume (0.5 cc) directly into three different parts of the tumor (intralesionaly). The yttrium-90 labeled antiferritin will only be given to the patients if the studies after indium-111 administration show that 90 percent or more of the deposited radioimmunoconjugates remains localized in tumor and draining lymph nodes. Blood and urine sample will be collected at 1, 20, 40, 120 and 165 hours after the yttrium-90 administration. The first 48 hours urine will also be collected. Bremsstrahlung scans will be performed at the same time points.

Procedures will be repeated twice with a week interval, and patients will receive a total activity of 0.3 mCi of yttrium-90 per gram of the tumor in three treatments over two weeks.

If five patients per histological tumor type have tolerated the procedure without acute side effects, the yttrium-90 activity will be escalated to 0.2 mCi per gram of tumor. If an additional five patients do not show acute side effects at the 0.2 mCi per gram of tumor level, an additional five patients will be done at the 0.4 mCi per gram of tumor level. Patients with Kaposi's Sarcoma will not receive more than 0.2 mCi of yttrium-90 per gram of tumor.

Statistical Considerations:

The study will be stopped if two out of five patients develop grade 3-4 acute side effects at any yttrium-90 level. If this happens for only one or two of the three tumor histologies under study, the study will continue in the uneffected tumor type(s).

Reagents:

The radioimmunoconjugate is prepared in four consecutive phases: Preparation of antiferritin antibody; Synthesis of immunoconjugate; Radiolabeling of immunoconjugate; Quality control of radioimmunoconjugate prior to clinical administration.

Preparation of Monoclonal Antibody:

Human ferritin isolated from a spleen of a patient with Hodgkin's disease was injected repeatedly with adjuvant in mice for monoclonal antibody production. After fusion, clones were screened. MDA 101 produced high affinity antiferritin. The clone was adjusted to hollow fiber culture and a batch of IgM was produced and purified. Antibodies were dispensed in single patient vials and checked for immunoreactivity activity (against ferritin), sterility and pyrogenicity.

Synthesis of immunoconjugate:

The isothiocyanatobenzyl - DTPA chelate is synthesized as described above.

Radiolabeling of the immunoconjugates:

The chelate conjugated antibody is maintained at 4°C until used. Radiolabeling is accomplished by adding to an (2 mg/ml) aliquot of immunoconjugate at room temperature purified carrier-free, freshly prepared $^{[1]}InCl_3$ that is dissolved in a mixture of acetate and citrate buffers at pH 5.5. Chelation of $^{[1]}In$ occurs within 30 minutes. The chelate is challenged by free DTPA in greater than 100 fold excess. The indium labeled immunoconjugates are purified on Sephadex G50 gel column chromatography (1.5 X 20 cm) using 0.05 M phosphate buffered saline. ^{90}Y radiolabeling is achieved by using a acetate buffer (2.0 M), pH 6.0 at room temperature similar to the $^{[1]}In$ chelation. ^{90}Y labeled immunoconjugates are purified on Sephadex G-100 column (1.5 X 30 cm) chromatography and eluted with 0.05 M PBS.

Quality Control Analysis of Radioimmunoconjugates:

The radioimmunoconjugate is filtered through 0.22 micron sterile filter and tested for lack of pyrogens by a Limulus Amebocyte Lysate (LAL) assay.

The purified antibody is tested for the presence of unconjugated $^{[1]}$In-DTPA by thin layer chromatography using a solvent mixture containing 10% ammonium acetate and methanol in 1 : 1 ratio (TLC silica) or 0.9% saline (ITLC silica). These solvent systems leave the labeled antibody at the origin and unconjugated $^{[1]}$In-DTPA at R_f1.0. The TLC and ITLC are completed before the administration of the antibody.

The radiolabeled immunoconjugates are also analyzed by size-exclusion HPLC to determine any colloid formation, and tested for radiochemical purity. The integrity of the radiolabeled antibody, prior to administration, is examined using HPLC. Analyses are performed using size-exclusion filtration Bio-silect SEC 250-5 (0.78 X 30 cm) column equilibrated in 100 mM sodium phosphate containing 150 mM sodium chloride, pH 6.8. Antibody samples (25 µl) are applied and the column runs at a flow rate 1.0 ml/min. The protein is detected by absorbance at 280 nm; fractions are collected at 1.0 min intervals and the radioactivity is measured in a gamma scintillation counter.

Patients:

Histological proof of Hodgkin's Disease, Kaposi's Sarcoma, or Head & Neck Caner. Patient must have measurable disease and failed prior potentially curative treatment regimens. Chemotherapy, surgery, radiation therapy or combinations. Potentially curative protocols will take precedent over this protocol.

Patient will be at least 10 years of age, with a life expectancy of over 3 months, and not pregnant. Patients will be advised to practice effective birth control during study.

Zubrod performance status 0, 1 or 2.

Patients must have adequate organ function as defined by: Granulocyte count $\geq$ 1500/mm^3; Platelet count $\geq$ 100,000/mm^3; Bilirubin $\leq$ 2.0, higher values acceptable if caused by HD or KS; Creatinine $\leq$ 2.0.Patients with abnormal serum chemistry or organ function can still be eligible for study, if this is due to their malignancy (primary or metastases).

Treatment:

Indium-111 labeled antiferritin IgM:

Under local anesthesia, three different needles (23 G) will be placed in three different parts of the tumor mass. Positioning of needle tip will be verified by two orthogonal X-rays films, real time ultrasound or CT. The selection of verification methods will depend on the anatomical location of the tumor. A volume of 0.5 cc containing 0.1 mg of antiferritin IgM labeled with 0.1 mCi of indium-111 will be utilized per injection site. Whole body scans will be obtained by gamma camera at 1, 20, 40, 120, and 165 hours after administration. A medium energy collimator will be utilized with collection centered at the peaks of indium-111 (173, 247 KeV). SPECT scans of tumor bearing area will be performed at 40 and 165 hours. Blood and urine samples will be obtained at 1, 20, 40, 120 and 165 hours and checked for radioactivity levels. The first and second 24 hours after administration, total urine will be collected for radioactivity testing. The percent injected dose in first and second twenty four hour urine, will be determined. The amount of radiation activity present in the tumor in one hour will be determined. Effective and biological half-life of the radioimmunoconjugate in the tumor will be determined, then elimination of blood radioactivity will be determined: monophasic, biphasic $T\alpha$, $T\beta$ and α/β. If over 90% of the deposited dose is retained in the tumor with a biological half-life of over 50 hours, patients will proceed to the intralesional administration of yttrium-90 labeled antiferritin.

Yttrium-90 labeled antiferritin IgM:

The volume of the tumor mass will be determined by summation of all transverse CT slices encompassing the tumor. The activity for administration will be 0.1 mCi per gram of the tumor (assuming the density of the tumor is 1). Administration will be performed as described under 5.1. The same studies will be performed after administration as given under 5.1., with the exception of using a low energy collimator and peaking of counts below 147 KeV. The results obtained will be compared to the results under 5.1 to verify as well as possible that the indium-111 labeled IgM has the same biodistribution and pharmacokinetics as the yttruim-90 labeled IgM.

The yttrium administration will be repeated twice, with one week intervals if the tumor remains detectable. For all histological types of tumor

an activity escalation will follow if the first five patients do not show acute side effects. New activity will be 0.2 mCI of yttrium-90 labeled antiferritin per gram of tumor per administration X 3. Only for Hodgkin's Disease and Head & Neck cancer patients will a third higher activity (0.4 mCi of yttrium-90 labeled antiferritin per gram of tumor X 3) be utilized if the second level (0.2 mCi X 3) does not produce acute side effects in five patients. The radiation dose per tumor is anticipated to be 30, 60 and 120 Gy for the 0.1, 0.2 and 0.4 mCi activity levels respectively.

Retreatment:

If a patient response is obtained or a complete response followed by recurrence, the patient can be retreated at the same or a higher activity level. If acute or late side effects have occurred at intensity level 3 or more after the first cycle, patients will not be retreated but go off study.

Evaluation during study:

Early side effects:

Allergic or anaphylactic reactions to low dose (2.5 mg) antiferritin radioimmunoconjugate have not been observed in the past. Vital signs of patients will be checked every 15 minutes for 60 minutes after injection of the radioimmunoconjugate. Anti-antibody formation has been observed in one patient only in prior studies of more than 80 patients with Hodgkin's Disease after I.V. antiferritin.[11] Anti-antibody (HAMA) is anticipated in patients with KS and Head & Neck Cancer. HAMA were noted in patients with glioblastoma multiforme and did not interfere with new cycles of treatment. HAMA will be determined two months after administration of antiferritin IgM and prior to a new cycle.

Late side effects:

Serum sickness or immune complex disease has not been observed in previously studied Hodgkin's Disease patients (n>80). The radiation dose received by normal tissues or Hodgkin's Disease tissues from indium labeled immunoconjugate is within the diagnostic X-ray/nuclear medicine range (less than 2 rad) and will not lead to biological effects. The yttrium labeled immunoconjugate is anticipated to cause tumoricidal effects and possibly

some acute or delayed side effects. Side effect evaluation is described under section 8.0 - Evaluation of Toxicity.

Tumor response:

Responses will be evaluated by repeated physical examinations, CT scans, gallium-67 scans and/or indium-111 antiferritin scans. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) will be used as endpoints and defined as complete disappearance of all prior known disease (CR), a fifty percent decrease or more in the product of orthogonal tumor diameters in old tumor sites with no appearance of new lesions (PR), less than fifty percent decrease or less than twenty-five percent increase in the product of orthogonal tumor diameters (SD), more than twenty-five percent increase in product of orthogonal tumor diameters (PD).

Evaluation of Toxicity:

All toxicity grades should be 1 or 0 prior to restarting a cycle. Grade 3 or 4 toxicity for any toxicity endpoints will prevent further treatment on this protocol for that particular patient and influence the treatment of other patients as indicated in Section 11.0. Toxicity grades will be assigned according to common toxicity grading criteria. After completion of treatment cycle, patients will be reevaluated in follow up once a month for two months. Thereafter follow up will be every two months X 2, followed by every three months X 2. Thereafter patients will be off study.

Criteria for Tumor response:

Acute and late side effects will be assessed. Tumor responses and duration will be determined by restaging methods, that will be selected for each patient and consist of physical exam and a selection of repeat diagnostic radiology studies (chest X-ray, CT scan, head and neck, chest, abdomen, pelvis, gallium scan, bone scan, indium-111 antiferritin scan). Only those diagnostic studies will be repeated that can provide unique information, instead of only duplicating information already known by other tests. In addition, the cheapest possible diagnostic test will be selected first.

Statistical Considerations:

Five patients will be tested at the first activity level. If therapy is well tolerated, escalation will be performed on the next five patients. If this escalation is tolerated, the remaining patients will be treated at the third radioactivity level. The third activity escalation will not be performed for the patients with Kaposi's Sarcoma.

Stopping rules are for toxicity. If toxicity exceeds grade 2 for two patients per group of five, that part of the study will be stopped.

Criteria for Removal From The Study:

Patients will be removed from study, in the case of progressive disease or toxicity of grade 3 or more. Patients removed from study will be followed for survival and late toxicity.

The teachings of the references cited herein are specifically incorporated herein. Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description and are intended to be encompassed by the following claims.

We claim:

1. A method for reducing the size of solid tissue comprising

administering an antibody composition intralesionally or intracompartmentally, wherein the antibody composition will remain localized in an amount effective to kill tissue at the site or within the compartment,

wherein the antibody composition comprises

antibodies selectively binding the tissue to be killed and which are in a form remaining localized in the tissue or region in which they are administered and

cytotoxic agents which are coupled to the antibodies.

2. The method of claim 1 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG, immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

3. The method of claim 1 wherein the cytotoxic agents are selected from the group of molecules consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

4. The method of claim 3 wherein the cytotoxic agents are cytotoxic radioisotopes.

5. The method of claim 3 wherein the cytotoxic agents are toxins.

6. The method of claim 2 wherein the antibodies are IgM.

7. The method of claim 1 wherein the antibodies comprise a conjugate which couples the cytotoxic agentss to the antibodies.

8. The method of claim 1 further comprising administering a diagnostic agent targeted to the same tissue as the cytotoxic agents, wherein the diagnostic agent is administered prior to administration of the cytotoxic agents, and detecting binding of the diagnostic agent to the tissue.

9. The method of claim 8 wherein the diagnostic agent is antibodies which selectively bind to the tissue to be killed labeled with a diagnostically detectable radioisotope.

10. The method of claim 1 wherein the tissue to be killed is solid tumor tissue.

11. The method of claim 10 wherein the tumors are selected from the group consisting of adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas.

12. The method of claim 1 wherein the tissue to be killed is endometriosis.

13. The method of claim 1 wherein the regions or compartments to be treated are selected from the group consisting of intrapleural compartments, head and neck cancer, breast, ovary, peritoneum, brain, and prostrate.

14. The method of claim 1 wherein the antibody composition is administered intraperitoneally.

15. The method of claim 1 wherein the antibody composition is administered intralesionally.

16. A method for treating disorders characterized by deposition of immune complex, inflammation and overproliferation of tissue comprising

administering at the site of immune complex deposition antibodies having cytotoxic agents coupled thereto.

17. The method of claim 16 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG, immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

18. The method of claim 16 wherein the cytotoxic agents are selected from the group of molecules consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

19. The method of claim 18 wherein the cytotoxic agents are cytotoxic radioisotopes and the disorder is rheumatoid arthritis.

20. An antibody composition comprising

antibodies selectively binding the tissue to be killed and which are in a form remaining localized in the tissue or region in which they are administered and

cytotoxic agents coupled to the antibodies.

21. The composition of claim 20 wherein the antibodies are selected from the group consisting of IgM, conjugates or polymers of IgG,

immunoglobulin fusion proteins, recombinant IgM fragments, and humanized IgM antibodies.

22. The composition of claim 20 wherein the cytotoxic molecules are selected from the group consisting of radioisotopes, toxins, substances which elicit the host to attack the tissue, chemotherapeutic drugs, oligonucleotides which interfere with cell growth or replication, and cytokines.

23. The composition of claim 22 wherein the cytotoxic agents are cytotoxic radioisotopes.

24. The composition of claim 22 wherein the cytotoxic agents are toxins.

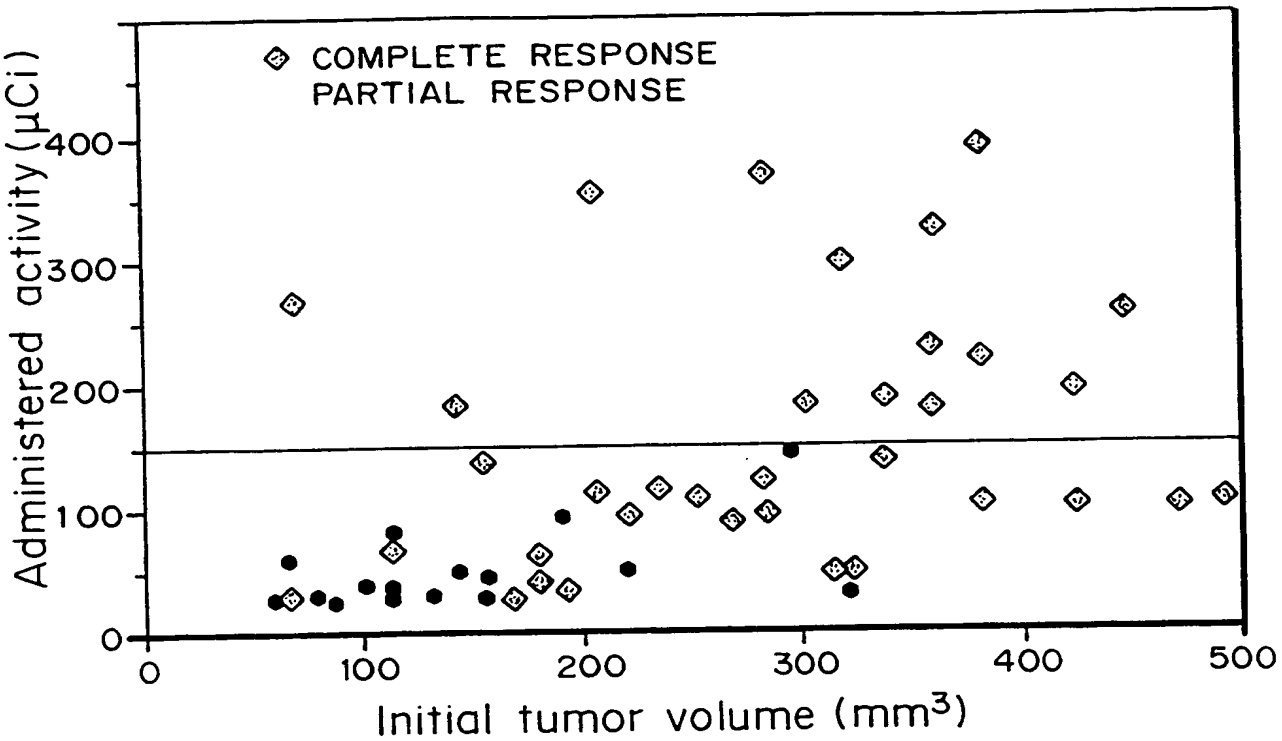
25. The composition of claim 20 wherein the antibodies are IgM.

26. The composition of claim 20 wherein the antibodies comprise a conjugate which couples the cytotoxic agents to the antibodies.

27. The composition of claim 26 wherein the conjugate is diethylenetriaminepentaacetic acid compound labelled with a radioisotope.

28. The composition of claim 20 in a kit further comprising a diagnostic agent targeted to the same tissue as the cytotoxic agents.

29. The composition of claim 20 wherein the antibodies are targeted to a tumor selected from the group consisting of adenocarcinomas, squamous cell cancers, sarcomas, glialblastomas, melanomas, neuralblastomas, and lymphomas.


COMPLETE RESPONSE
PARTIAL RESPONSE
Administered activity (μCi)
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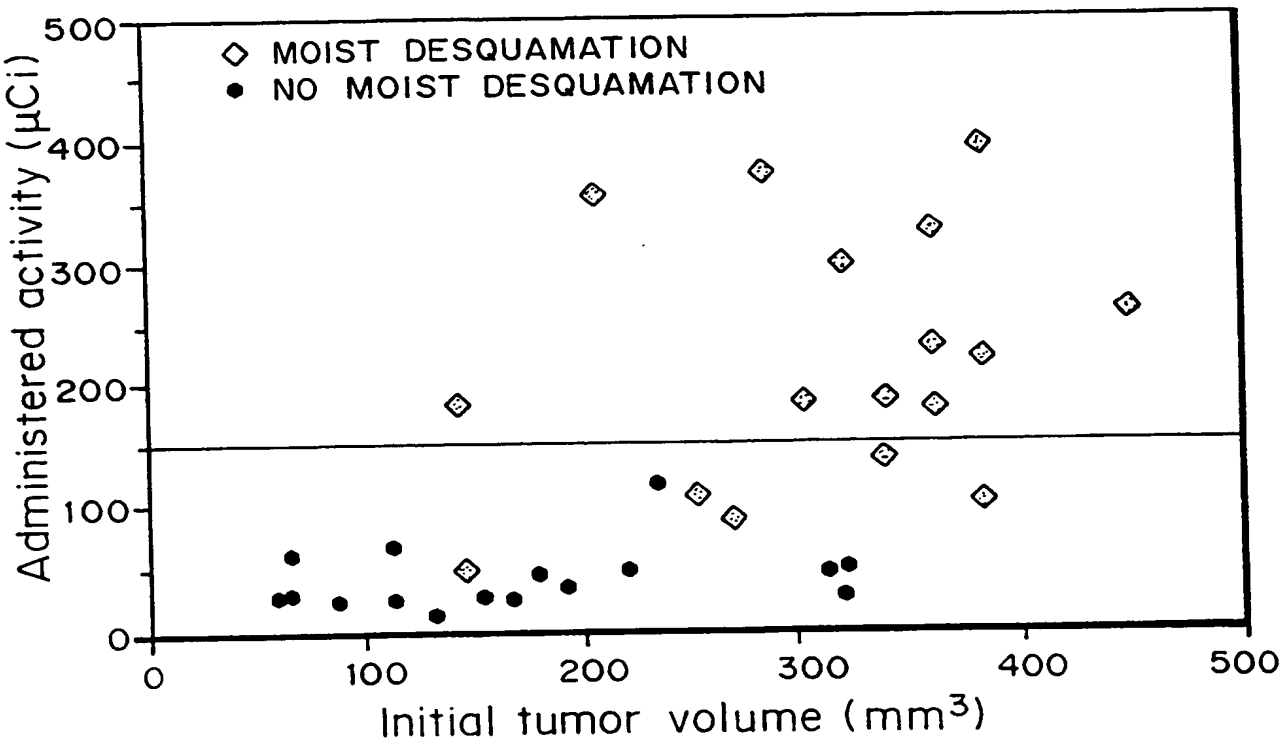
100
200
300
400
0
100
200
300
400
500
Initial tumor volume (mm3)

FIG. 1


MOIST DESQUAMATION
NO MOIST DESQUAMATION
Administered activity (μCi)
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100
200
300
400
500
0
100
200
300
400
500
Initial tumor volume (mm3)

FIG. 2

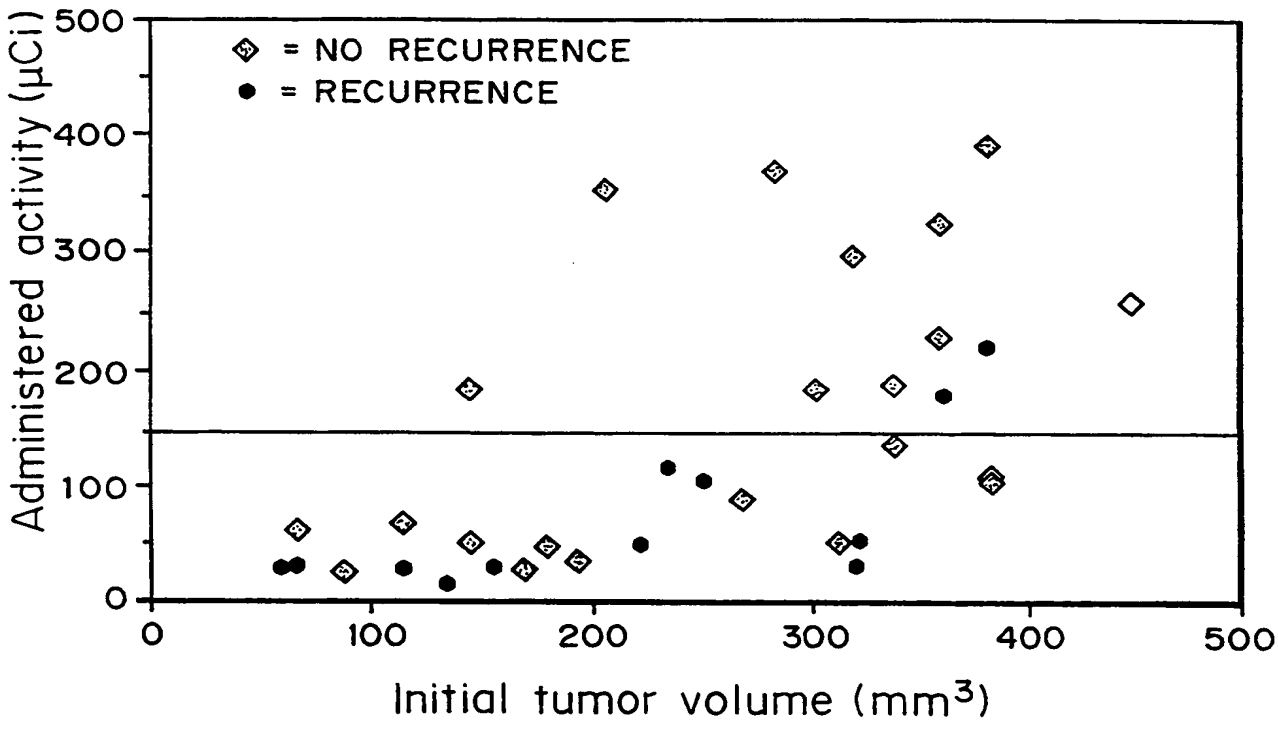

FIG. 3

CORRECTED VERSION*

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(57) Abstract

It has been discovered that large antibody aggregates or molecules, such as IgM or conjugated IgG or IgG fusion proteins, can be used to treat tumors by intracompartmental or intratumoral administration of anti-tumor antibody coupled to a toxin. The method can also be used in the treatment of certain disorders characterized by deposition of immune complex, for example, rheumatoid arthritis. In the preferred embodiment, the antibody is IgM and the toxin is a radioisotope, most preferably ^{111}In-labelled IgM or ^{90}Y-labelled IgM. Examples demonstrate effectiveness in mice models.

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CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00857

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K51/10 A61K47/48 A61K31/385
//A61K103:32,A61K131:00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORCHARDT P.E. ET AL: "Intralesional radiolabeled human monoclonal IgM in human tumor xenografts" RADIOTHERAPY AND ONCOLOGY, 1997, VOL. 44, NO. 3, PAGE(S) 283-293, XP002112294 see abstract paragraph [MATERIALS AND METHODS] see paragraph RESULTS --- -/--	1-4, 6-11, 13-18, 20-23, 25-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 1999	10. 12. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	DULLAART A.W.M.

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00857

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE DISSERTATION ABSTRACTS University Microflims International BORCHARDT, PAUL ERIC: "RADIOLABELED HUMAN MONOCLONAL IGM FOR INTRACOMPARTMENTAL CANCER THERAPY (RADIOIMMUNOTHERAPY, INDIUM, YTTRIUM, LYMPHOMAS)" XP002112299 see abstract & DISSERTATION ABSTRACTS INTERNATIONAL, vol. 58, no. 08 part B, 1997, page 4177 & "Ph. D Thesis" 1997 , THE UNIVERSITY OF TEXAS H.S.C. AT HOUSTON GRAD. SCH. OF BIOMED. SCI , HOUSTON, US ---	1-4, 6-11, 13-18, 20-23, 25-29
X	QUADRI S M: "Intraperitoneal In-111 and Y - 90 labeled human IgM (AC6C3 -2B12) in nude mice with peritoneal carcinomatosis of a human cancer." JOURNAL OF NUCLEAR MEDICINE, 1995, VOL. 36, NO. 5, PAGE(S) 160P, XP002112295 abstract No. 729 see abstract ---	1-4, 6-11, 13-18, 20-23, 25-29
X	QUADRI, SYED M. ET AL: "Preclinical analysis of intraperitoneal administration of 111In-labeled human tumor reactive monoclonal IgM AC6C3-2B12" CANCER RES., 1995, VOL. 55, NO. 23, SUPPL., PAGE(S) 5736S-5742S, XP002112296 see abstract see paragraph RESULTS see paragraph RESULTS; figure 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
X	QUADRI, SYED M. ET AL: "Intraperitoneal indium-111- and yttrium-90-labeled human IgM (AC6C3-2B12) in nude mice bearing peritoneal carcinomatosis" J. NUCL. MED., 1996, VOL. 37, NO. 9, PAGE(S) 1545-1551, XP002112297 see abstract; figure 1 see page 1545, right-hand column; figure 1 see page 1546, right-hand column - page 1547, left-hand column; figure 1 see paragraph RESULTS; figure 1 see paragraph CONCLUSI; figure 1 --- -/--	1-4, 6-11, 13-18, 20-23, 25-29

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00857

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BORCHARDT PE ET AL: "Indium-111- and yttrium-90- labeled human monoclonal immunoglobulin M targeting of human ovarian cancer in mice." J NUCL MED, MARCH 1998, VOL. 39, NO. 3, PAGE(S) 476-484, XP002112298 see abstract; figure 1 see abstract; figure 1; table 1 see paragraph RESULTS; figure 1; table 1 see paragraph CONCLUSI; figure 1; table 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
X	WO 90 15625 A (AKZO NV) 27 December 1990 see page 12, last paragraph - page 15; figure 1; table 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
X	WO 97 44461 A (DAN MICHAEL D ;KAPLAN HOWARD A (CA); MAITI PRADIP K (CA); NOVOPHAR) 27 November 1997 see page 12, last paragraph - page 15; examples 2,3,11; table 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
X	WO 93 17707 A (AKZO NV) 16 September 1993 see page 12, last paragraph - page 15; examples 1-3; table 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
Y	US 5 583 219 A (SUBRAMANIAN RAMASWAMY ET AL) 10 December 1996 see page 12, last paragraph - page 15; example 1; table 1 ---	1-4, 6-11, 13-18, 20-23, 25-29
X	BENACH J L ET AL: "A MURINE IGM MONOCLONAL ANTIBODY BINDS AN ANTIGENIC DETERMINANT IN OUTER SURFACE PROTEIN A, AN IMMUNODOMINANT BASIC PROTEIN OF THE LYME DISEASE SPIROCHETE" JOURNAL OF IMMUNOLOGY, vol. 140, no. 1, 1 January 1988, pages 265-272, XP002001274 see page 267; example 1; table 1 see page 267; example 1; table 1 see page 267; figures 3,4; table 1 --- -/--	1-4, 6-11, 13-18, 20-23, 25-29

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00857

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RIVA P ET AL: "LOCOREGIONAL IMMUNOTHERAPY OF HUMAN OVARIAN CANCER: PRELIMINARY RESULTS" INTERNATIONAL JOURNAL OF RADIATION APPLICATIONS AND INSTRUMENTATION PART B: NUCLEAR MEDICINE AND BIOLOGY, vol. 16, no. 6, 1 January 1989, pages 659-666, XP000054551 see paragraph CONCLUSI; figures 3,4; table 1 see paragraph CONCLUSI; figures 2,3; table 1 see paragraph SUMMARY; figures 2,3; table 1 ---	1-4
Y	WATANABE N ET AL: "Preparation of Yttrium-90-Labeled Human Macroaggregated Albumin for Regional Radiotherapy" NUCLEAR MEDICINE AND BIOLOGY, vol. 24, no. 5, 1 July 1997, page 465-469 XP004085841 see paragraph INTRODUC; figures 2,3; table 1 -----	1-4, 6-11, 13-18, 20-23, 25-29

1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/00857

B x I Observations where ertain claims were found unsear hable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4,6-11,13-18,20-23,25-29 in part

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-4, 6-11, 13-18, 20-23, 25-29 in part

Antibody compositions in which the antibody is coupled to a cytotoxic radioisotope, and their use in cancer therapy.

2. Claims: 1-3, 5-11, 13-18, 20-22, 24-26, 28, 29 in part, and 5, 24

Antibody compositions in which the antibody is coupled to a toxin, and their use in cancer therapy.

3. Claims: 1-3, 6-11, 13-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a substance which elicits the host to attack the tissue, and their use in cancer therapy.

4. Claims: 1-3, 6-11, 13-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a chemotherapeutic drug, and their use in cancer therapy.

5. Claims: 1-3, 6-11, 13-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to an oligonucleotide which interferes with cell growth or replication, and their use in cancer therapy.

6. Claims: 1-3, 6-11, 13-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a cytokine, and their use in cancer therapy.

7. Claims: 1-4, 6-18, 20-23, 25-29 in part

Antibody compositions in which the antibody is coupled to a cytotoxic radioisotope, and their use in the therapy of endometriosis.

8. Claims: 1-3, 5-18, 20-22, 24-26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a toxin, and their use in the therapy of endometriosis.

9. Claims: 1-3, 6-18, 20-22, 25, 26, 28, 29 in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Antibody compositions in which the antibody is coupled to a substance which elicits the host to attack the tissue, and their use in the therapy of endometriosis.

10. Claims: 1-3, 6-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a chemotherapeutic drug, and their use in the therapy of endometriosis.

11. Claims: 1-3, 6-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a oligonucleotide which interferes with cell growth or replication, and their use in the therapy of endometriosis.

12. Claims: 1-3, 6-18, 20-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a cytokine, and their use in the therapy of endometriosis.

13. Claims: 1-4, 6-11, 13-23, 25-29 in part

Antibody compositions in which the antibody is coupled to a cytotoxic radioisotope, and their use in the therapy of rheumatoid arthritis.

14. Claims: 1-3, 5-11, 13-22, 24-26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a toxin, and their use in the therapy of rheumatoid arthritis.

15. Claims: 1-3, 6-11, 13-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a substance which elicits the host to attack the tissue, and their use in the therapy of rheumatoid arthritis.

16. Claims: 1-3, 6-11, 13-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a chemotherapeutic drug, and their use in the therapy of rheumatoid arthritis.

17. Claims: 1-3, 6-11, 13-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to an

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

oligonucleotide which interferes with cell growth or replication, and their use in the therapy of rheumatoid arthritis.

18. Claims: 1-3, 6-11, 13-22, 25, 26, 28, 29 in part

Antibody compositions in which the antibody is coupled to a cytokine, and their use in the therapy of rheumatoid arthritis.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 3.

Although claims 1-4, 6-11 and 13-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Further defect(s) under Article 17(2)(a):

Continuation of Box 3.

Claims Nos.: 1-4, 6-11, 13-18, 20-23, 25-29 in part

The initial phase of the search revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved (in fact, the definition of claims 1 and 20 comprise the IPC subgroup A61K51/10 in its entirety) that it is impossible to determine which parts of the claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of the claim(s) is impossible. Consequently, the search has been restricted to the compositions based on the specific antibodies mentioned in the description in table 1.

Although not encompassed by the present search it is already pointed out, that endometriosis is not a tissue, but a disease. Therefore, claim 12 is not correct.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/00857

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9015625 A	27-12-1990	AT 102837 T	15-04-1994
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